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Women

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## 5. INTRODUCTION

### a. Overview

The overall purpose of this project is to investigate interactions between bacteria which commonly cause urinary tract infection (UTI) and their cognate host cell receptors in the vaginal and bladder epithelium in order to design novel, non-antibiotic methods for preventing UTIs. The project is focused on studying the two most common uropathogens causing UTI in young women, Escherichia coli and Staphylococcus saprophyticus, as well as their interactions with glycosphingolipids (GSLs) on the cell surface of the bladder and vagina. In the first three years of this project, we will define the key GSLs on the eukaryotic cell surface that uropathogenic bacteria use for attachment and then in the last two years, we will take advantage of new biochemical techniques using carbohydrate mimetics to design UTI prevention methods that avoid the induction of antimicrobial resistance. This report describes progress made in the fourth year of this grant. It is an annual report because the project is presently in a no-cost extension period.

### b. Background presented in original proposal

Acute uncomplicated UTIs caused by E. coli and S. saprophyticus occur in an estimated 7 million young women each year at an annual cost for diagnosis and treatment exceeding one billion dollars. Over half of all women have had a bacterial UTI by their late 20's and approximately 20% of women with UTI suffer very frequent ( $\geq 3/\text{year}$ ) recurrences (1). Nonetheless, the only currently available preventive modality for these recurrent infections is antimicrobial prophylaxis. Though effective, antimicrobial prophylaxis may promote the emergence of drug-resistant strains (1). In addition, women typically revert to having frequent recurrences once prophylaxis ceases and little is known about why some women suffer frequent recurrences of UTI, since this phenomenon cannot usually be explained by underlying functional or anatomic abnormalities of the urinary tract (1). The interaction of infecting bacterial strains with the women's epithelial cells appears to be a critical point in the infectious process that determines host susceptibility, in particular the availability and nature of host cell bacterial ligands such as GSLs (2-4).

GSLs are important components of the glycocalyx surrounding mammalian cells and consist of an oligosaccharide moiety exposed on the cell surface, to which organisms attach, covalently linked to a lipid portion embedded in the outer leaflet of the plasma membrane. They serve as eukaryotic cell adhesion sites for many pathogens and their toxins, including E. coli, Pseudomonas aeruginosa, Helicobacter pylori, HIV, parvovirus, rotavirus, cholera toxin, verotoxin of E. coli 0157, and others (5-9). Based on the structures of their carbohydrate components, they are grouped into families, such as the lacto- and neolacto-series, the globoseries, and the ganglioseries GSLs. GSLs are synthesized by the sequential action of glycosyltransferases, many of which are tissue-specific and/or genetically determined (10). Thus, GSLs on the cell surface play an important role both in determining tissue tropism and an individual host's susceptibility to specific infectious diseases.

Among uropathogenic E. coli, isolates expressing the pap-encoded family of adhesins are significantly overrepresented among strains collected from patients with UTI as compared with fecal isolates from patients without UTI (11). The GSL receptors for these adhesins in the kidney are the globoseries GSL family that contain a minimal receptor consisting of a galactose  $\alpha$ -1-4 galactose moiety (11). Although S. saprophyticus is the second most common cause of UTI, to our knowledge, we are the first to actually investigate whether it binds to GSLs. In our grant proposal, we showed preliminary data demonstrating that the wild-type S. saprophyticus strain ST352 binds asialo GM1 (ASGM1), a neutral ganglioseries GSL and that other wild-type S. saprophyticus isolates bind to ASGM1 and/or structurally-related ganglioseries GSLs. Paradoxically, while globoseries GSLs have been identified in kidney tissue and vaginal epithelium, the bladder has been little studied with respect to GSLs, despite the fact that it is the most common site of UTI. Previous studies of GSLs in native bladder tissues have focused on oncogenesis or development and have not included GSLs that are directly involved in adhesion of uropathogens (ganglioseries and globoseries GSLs). Of note, other urogenital pathogens, including C.

albicans, C. trachomatis, N. gonorrhoeae, have also been reported to bind to ASGM-1 and it is possible that a single class of inhibitors could prevent adherence and infection with all of these agents.

### **c. Culture and characterization of vaginal epithelial cells**

#### The native vaginal epithelium in vivo and in vitro

As a mucosal surface, the vagina serves as a barrier to local infection, but since it is situated at the entrance to the upper genital tract, it also serves protective functions. It is a non-keratinized, stratified squamous epithelium lacking glands. Based on cellular morphology by light microscopy, the epithelium is divided into several layers: the basal/proliferative, parabasal, intermediate, and superficial layers. A complex ecosystem exists within the vagina of post-menarchal women, consisting of at least 50 species of bacteria, but dominated by lactobacilli, which appear to have a protective effect in this microniche (2, 7, 18). Conversely, increased vaginal colonization with E. coli in young, otherwise healthy women is associated with increased risk of UTI (45), and during pregnancy, colonization with E. coli is associated with preterm birth and other perinatal complications (18, 34).

Hormonal influences such as the onset of menopause or the use of hormone replacement therapy is associated with changes in thickness of the vaginal mucosa and alterations in the proportion of cells demonstrating basal and parabasal morphologies. There is a loss of lactobacillus colonization and an increase in colonization with potential pathogens such as E. coli, Group B streptococcus, and enterococci (38). However, perhaps because of the paucity of in vitro model systems, there have been few studies of the effects of exogenous sex hormones on nearly any aspect of the basic biology of the vaginal epithelium, including markers of differentiation, bacterial attachment and colonization with normal flora. As an extension of the original aims of this proposal, we have been developing and characterizing a model system of vaginal epithelium. When we first proposed these studies, we planned to use vaginal epithelial cell cultures established by a collaborator. For practical reasons, we established our own culture system subsequently and we then found that the literature contained little information about the biology of vaginal epithelial cells grown in culture. Our efforts to systematically characterize our culture system in a manner analogous to procedures used by keratinocyte biologists have led us to new avenues of investigation of hormonal and other effects, as described in detail below.

#### Keratins in vaginal tissue

Our initial approach to characterizing the cultured vaginal epithelial cells has been to define keratin expression in our cultured cell. Keratins are key markers of cell origin and differentiation in stratified squamous epithelium. Their expression has been more thoroughly studied in other stratified squamous epithelium such as the mouth. A review of these studies as well as studies of native human and mouse vaginal tissue suggests patterns of keratin expression correlating with specific cell layers and with morphological degrees of differentiation have been developed. K19, generally associated with epithelial proliferation, has been found in basal layers of human vaginal epithelium (14). K13 has been used as a marker of suprabasal cell layers and thus of differentiation (42, 43). In the mouse, K14 is found in all layers of the epithelium and K1 was not expressed in undifferentiated epithelium but was upregulated in response to exogenous estrogen (15). Human vagina stained in all layers for K10, usually associated with the epidermis in the skin (14).

### **d. Brief summary of preliminary data presented in original proposal**

Our original proposal presented preliminary data demonstrating that primary cultures of human bladder epithelial cells are a promising model system for the study of bladder GSLs in the pathogenesis of UTI caused by E. coli or S. saprophyticus. Specifically, we showed that these cell cultures appear to express globoseries GSLs, the host cell binding ligand for an important class of uropathogenic E. coli, those expressing pap-encoded adhesins. We previously showed that SGG and other globoseries GSLs are surface exposed in human kidney tissues and exfoliated vaginal epithelial cells (2). In our preliminary

immunocytology experiments with primary cultures of human uroepithelium, the cells demonstrated bright immunofluorescent staining with MAb ID4 directed against SGG, suggested that this epitope is also surface exposed on these bladder cells. We also showed that S. saprophyticus, the second most common cause of UTI in young women, binds to ganglioseries GSLs, especially ASGM1 and ASGM2. In addition, we demonstrated the presence of ASGM1 among GSLs extracted from human kidney and the surface exposure of this epitope in kidney sections in specific histological areas where bacteria also adhere. Another ganglioseries GSL, GM1, was identified among GSLs extracted from human kidney tissues and vaginal epithelial cells. ASGM1 appears to be surface exposed on both kidney and cultured primary bladder cell surfaces, as shown by positive immunofluorescent staining with MAb TKH-7, directed against ASGM1.

#### **d. Originally proposed hypotheses**

The original hypotheses of this project have been supported by data obtained during the first two years of the grant and thus remain unchanged, as listed below. The overall goal of this project remains to define the key eukaryotic cell surface GSLs that are used by uropathogenic bacteria for attachment and then to take advantage of new biochemical techniques utilizing carbohydrate mimetics to design novel means for preventing UTIs that avoid the use of antimicrobials.

- (1) We hypothesize that globoseries and ganglioseries GSLs are present in primary cultures of bladder transitional epithelium and vaginal epithelium and serve as binding sites for E. coli and S. saprophyticus, respectively.
- (2) We hypothesize that the GSLs identified in the first hypothesis are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.
- (3) We hypothesize that carbohydrate mimetic and synthesis techniques can be used to design high-affinity inhibitors of E. coli and S. saprophyticus binding to vaginal and bladder transitional epithelium.

#### **e. Original technical objectives**

During the third year of funding for this project, we did not alter any of our technical objectives, but we increased the emphasis on certain aspects of our objectives. We expanded previously established collaborations and developed new ones, some of which promise to open new areas of inquiry in the coming year. For some of the objectives, we used slightly different but more efficient technical approaches. These changes are discussed in greater detail below. Our technical objectives are as follows:

- (1) We will extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional epithelium and vaginal epithelium, according to the following sequence: (a) purify the GSLs using high-performance liquid chromatography (HPLC); (b) identify bacteria-binding GSLs by overlaying radiolabeled isolates of E. coli and S. saprophyticus on these GSLs separated on high-performance thin-layer chromatography (HPTLC); (c) confirm the identities of these GSLs using specific monoclonal antibodies (MAbs) directed against the GSLs in HPTLC immunostaining assays; and (d) perform carbohydrate structural analysis on the bacteria-binding GSLs.
- (2) To demonstrate that the GSLs identified in Hypothesis 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection, we will: (a) test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures; (b) utilize immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs; (c) repeat 2a and 2b after pretreatment of the cell cultures with an inhibitor of GSL receptor synthesis; and (d) repeat 2a after pretreatment of the cell cultures with the MAbs directed against relevant GSLs.



(3) We will use carbohydrate mimetic techniques to design inhibitors of bacterial adherence, focusing initially on the interaction of E. coli with sialosyl galactosyl globoside (SGG, a GSL to which E. coli binds with high affinity; see preliminary data). We will test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays, as described in the second objective.

## **6. BODY OF REPORT**

### **A. Overview**

#### **1. Review of changes and opportunities occurring in the first two years of funding:**

(1) Establishment of a new collaboration with Dr. Steven Levery of the Complex Carbohydrate Research Center (CCRC) of The University of Georgia, Athens: We continue to work with Dr. Levery for assistance in structural characterization of GSLs (see results).

(2) Establishment of a collaboration with Drs. M. Juliana McElrath and Florian Hladik of the Fred Hutchinson Cancer Research Institute and the University of Washington: Though this project has ended, but the technical aspects and opportunities associated with this work has been replaced this new collaborations involving members of the Department of Obstetrics and Gynecology. (see below).

(3) Vaginal epithelial cell cultures: This aspect of the project has become a major focus of the project in the past year, through expansion of technical knowledge, development of new collaborations and protocols for tissue acquisition, and the development of new hypotheses. These are detailed below.

(4). New approaches taken by Dr. Stroud: Dr. Stroud has further expanded new approaches developed in the last funding period for accomplishing Technical Objective 3, detailed below.

#### **2. New opportunities and approaches during the third year of funding:**

1. Bladder cell cultures: we have begun collaborating with Dr. Richard Grady, Assistant Professor of Urology at the University of Washington and Children's Hospital and Regional Medical Center. Dr. Grady has assisted us in obtaining urothelial tissue and in augmenting our culture stocks.

2. Meetings with collaborators based outside Seattle:

a. Dr. Atala: Dr. Stapleton met with Dr. Atala in May 1999 and plans another meeting in November 1999.

b. Dr. Levery: Dr. Stroud met with Dr. Levery during several visits by Dr. Levery to Seattle.

c. Dr. Toyokuni: Dr. Stroud will meet with Dr. Toyokuni in November at a West Coast glycobiology meeting this fall.

#### **3. New opportunities and approaches during the fourth year of funding:**

1. We have initiated a collaborative effort with Dr Beverly Dale-Crunk, Professor of Oral Biology and Scientific Director of the Comprehensive Oral Research Center at the University of Washington to characterize keratin expression in cultured vaginal epithelial cells.

2. In collaboration with members of the Mass Spectrometry Laboratory in the Department of Medicinal Chemistry at the University of Washington, we have instituted the use of matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) technology to characterize GSLs isolated from bladder epithelial cells. The Mass Spectrometry Center is an instrument laboratory providing a wide variety of mass spectral services to the University, offering both analytical and instructional expertise in mass spectrometric

techniques and instrumentation, with the primary aim of providing investigators with "hands-on" training in the use, as well as, ready access to the modern instrumentation necessary in carrying out their research goals.

**4. Other issues:** We are presently in a period of no-cost extension because of personnel issues that have left us with some of the objectives yet to be met in the coming year. Dr. Stroud's group moved its laboratory twice in the past year, a great inconvenience which caused him to lose considerable time. He is now housed in a newly renovated facility which will offer many advantages.

## B. Original Statement of Work

The original technical objectives set for all four years of funding are listed below.

**Technical Objective 1:** Extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional and vaginal epithelium.

- |                |                                                                                        |
|----------------|----------------------------------------------------------------------------------------|
| <b>Task 1:</b> | Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells |
| <b>Task 2:</b> | Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures              |
| <b>Task 3:</b> | Months 7 to 12: bacterial overlay assays                                               |
| <b>Task 4:</b> | Months 7 to 12: immunostaining assays                                                  |
| <b>Task 5:</b> | Months 13 to 24: carbohydrate structural analysis                                      |
| <b>Task 6:</b> | Months 25 to 36: data analysis and publication                                         |

**Technical Objective 2:** Demonstrate that the GSLs identified in Technical Objective 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

- |                |                                                                                                                                                     |
|----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Task 1:</b> | Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures   |
| <b>Task 2:</b> | Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs |
| <b>Task 3:</b> | Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays                      |
| <b>Task 4:</b> | Months 24 to 36: MAb pre-treatment, followed by GSL extraction and quantification and bacterial adherence assays                                    |
| <b>Task 5:</b> | Months 30 to 36: data analysis and publication                                                                                                      |

**Technical Objective 3:** Use carbohydrate mimetic techniques to design inhibitors of bacteria adherence, focusing initially on the interaction of E. coli with SGG; test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays.

- |                |                                                                                                                                                                                 |
|----------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Task 1:</b> | Months 24 to 42: synthesis of linear mimetic compounds                                                                                                                          |
| <b>Task 2:</b> | Months 27 to 42: structural analysis of resulting compounds (procedures to begin as each new compound is prepared)                                                              |
| <b>Task 3:</b> | Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial overlay assays, focusing on SGG as the target GSL to which binding should be inhibited |

- Task 4:** Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial adherence assays, using primary cultures of bladder and vaginal epithelial cells
- Task 5:** Months 30 to 48: synthesis of multivalent structures from linear structures that are effective in Tasks 3 and 4
- Task 6:** Months 40 to 48: repeating Tasks 3 and 4 using multivalent compounds
- Task 7:** Months 40 to 48: data analysis and publication

### C. Details of Progress

#### 1. Technical Objective 1

##### a. Task 1, Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells

##### 1. Experimental methods, assumptions and procedure

Primary cultures of human bladder epithelial cells were provided by Dr. Anthony Atala and maintained in serum free keratinocyte media using standard tissue culture techniques, as in our preliminary studies and as he has described (13). Briefly, cells were maintained in serum- and antibiotic-free keratinocyte medium and passed at 70% confluence in a ratio of 1:4 or 1:6 (13). Each time cells were passed, the number of flasks were maximally expanded to prepare cells for the purification of GSLs of interest. This procedure was continued with each cell sample until senescence was noted, usually at about passage 12.

In addition, we established primary cultures of bladder epithelial cells in our own facility, with the assistance of Dr. Richard Grady. To accomplish this, we adapted the methods developed for culture of primary vaginal epithelial cells, with one change. Specimens from bladder epithelium are generally processed in the operating room, rather than transporting them back to our facility first. Cultures are maintained as described above.

The collaboration we established with Drs. McElrath and Hladik during the first two years of funding resulted in our establishing a technique of maintaining primary cultures of vaginal epithelial cells in laboratory. The parent project for this collaboration has since ended, thus we replaced it with a project of our own. We added several new collaborators, listed below. Details of how these collaborations have assisted us in our investigations are noted in the results and recommendations sections for the relevant tasks below.

##### (1) Member of the Department of Obstetrics and Gynecology:

**a. David Eschenbach, MD**, Professor of Obstetrics and Gynecology, University of Washington, was a key contact person in the original project and has been a collaborator of ours on other projects for many years. He has continued to provide discarded vaginal tissue specimens for use in our experiments. Along with Dr. Patton, he has consulted on issues regarding investigating estrogen stimulation of our primary vaginal epithelial cells lines, in order to better approximate the biology of the vaginal epithelium.

**b. Dorothy Patton, PhD**, Professor of Obstetrics and Gynecology and Adjunct Professor, Biological Structure and Ophthalmology, University of Washington. Dr. Patton has assisted us with issues specific to culturing vaginal epithelium and with immunohistostaining these tissues. Since she has years of prior experience with genital tissue-derived cultured cells, she has assisted us with assessing the effects of hormonally stimulating our vaginal epithelial cell cultures.

c. **Drs. Dee Fenner** (Associate Professor) and **Gretchen Lentz** (Assistant Professor) have also assisted us with acquisition of appropriate discarded vaginal tissue for our studies and have also provided clinical correlation with hormone-related issues.

(2) **Beverly Dale-Crunk, PhD**, Professor of Oral Biology and Adjunct Professor, Medicine (Dermatology), Periodontics, and Biochemistry at the University of Washington. Dr. Dale has assisted us in determining markers of epithelial differentiation and in assessing epithelial morphological changes in response to estrogen during this past year of funding. We are in the early stages of preparing these data for publication.

## 2. Results and discussion

Primary bladder epithelial cells: We are continuing to culture primary bladder epithelial cells from samples obtained through Dr. Grady at Children's Hospital in Seattle, to augment our work with Dr. Atala. We have maintained our collaboration with Dr. Atala in order to compare the results obtained in our hands, using his techniques, with cell established in his laboratory, with his established methods. This funding year, we have processed 14 samples, with the following outcomes:

Number of samples	Outcome
5	Cell line established; pellets obtained for GSL extraction; stocks of these lines established in liquid nitrogen
5	Tissue never attached and was discarded
0	samples were combined with one another by accident and were discarded
2	Contaminated before establishment
2	recently established cultures; results pending

Primary vaginal epithelial cells: In the past year of funding, we processed 18 samples, with the following results:

Number of samples	Outcome
5	Cell line established; pellets obtained for GSL extraction; stocks of these lines established in liquid nitrogen
8	Cell line established, but insufficient material for storage of stocks
5	Contaminated before establishment

Further progress in the characterization of the largest vaginal epithelial cell pellet described last year is detailed below. We also describe characterization of GSLs extracted from a 6 ml pellet of bladder epithelial cells.

Details of technical progress in culture efforts are as follows:

a. General: Issues regarding seeding, avoidance of contamination, and eliminating fibroblast contamination: these techniques continue to be routine in the laboratory now, and no unusual problems have resulted this past year. We have also further adapted our methods for large surface area cultivation to obtain larger cell pellets of both types of cells for GSL characterization.

b. Cryopreservation: Our collection of cryopreserved cells has been steadily augmented, and methods for re-seeding frozen cultures have been maximized. We have been able to revive cultures preserved over one year ago.

### c. Characterization of cultured vaginal epithelial cells

Immunohistostaining: We used a standard immunohistostaining procedure to confirm the epithelial origin of our cultures. We tested cultures derived from five separate individuals at first to fifth passage by staining with a MAb (ascites) which stains all epithelial cells (PAN; Sigma) and with a MAb directed against fibroblasts (anti-fibroblast antibody; Sigma). We used HeLa cells and T24 bladder epithelial cells (ATCC) as positive controls for the PAN antibody staining. Pure cultures of fibroblasts from vaginal epithelium (derived from early, failed attempts at culturing VECs) as well as McCoy mouse fibroblast cells were used as positive controls in the anti-fibroblast antibody assays. Cultures of VEC cultures from all five individuals produced a strongly positive signal with the PAN MAb and no signal with the anti-fibroblast antibody.

Characterization of differentiation markers: To investigate the expression of keratins under the cell culture conditions previously investigated, we tested cells grown in SFM and PRF-SFM, with and without exogenous 17- $\beta$ -estradiol, and differentiated by the addition of serum.

We have completed the first phase of our experiments performed in collaboration with Dr. Beverly Dale to characterize the expression of keratins in cultured vaginal epithelial cells; results are presented below.

Keratins: Based upon our discussions with Dr. Dale and our review of the literature, we investigated the expression of a panel of keratins that are predicted to stain differentially in cultured VECs or tissue sections, and/or their expression may be regulated by estrogen exposure: (a) K19, expected to be found expressed in basal cells; (b) K14, likely to be found in cells of varying degrees of differentiation; (c) K13, expected to be expressed in suprabasal cells; (d) K10, described as appearing in a mosaic pattern in native vaginal epithelium (28, 29); and (e) K1, a keratin whose expression is regulated by estrogen in vivo and which has also been seen in a mosaic pattern in native tissue (28, 29). We also have recently begun to study the expression of K6 and K16, usually associated with rapidly turning over epithelia, wound healing, growth in cell culture, and some hyperproliferative disorders of epidermis, and normally found in palatal and gingival epithelia.

Protein extraction: We extracted keratins from the V26 vaginal epithelial cell culture, using protein extraction methods designed to enrich for keratins to extract one of the VEC cell lines and performed Western blots using the PAN antibody. Briefly, equal volumes of cultured cells were harvested using trypsin, then cells were washed in PBS and extracted at 4° C in 6 M urea/10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 5 mM EDTA, 5 mM dithiothreitol, and the protease inhibitor 1 mM phenylmethylsulfonyl fluoride (40). Extracts were stored at -20° C until use.

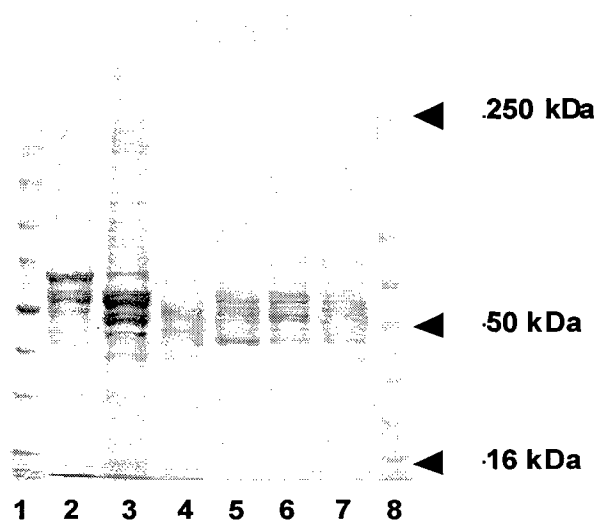
SDS-Gel Electrophoresis and Immunoblotting : One-dimensional SDS- polyacrylamide gradient gels (8-16%) were run according to Laemmli (41). Total protein concentration was determined using a commercial method (Biorad) and samples were normalized so that each lane was loaded with 15  $\mu$ g protein. Samples were boiled for 5 min with 2% SDS and 10% 2-mercaptoethanol before loading for electrophoresis. Proteins were stained with Coomassie Brilliant Blue. Proteins from duplicate unstained gels were transferred electrophoretically to nitrocellulose membrane using a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, for 1 h in Tris-glycine buffer, pH 7.5, with 20% methanol). Membranes were blocked for 1 h in Tris Buffered Saline with 0.05% Tween (TBST) containing 5% dried milk to block additional protein binding sites, then incubated sequentially in monoclonal antibody at 1: 100 to 1: 500 dilution overnight at +4°C, goat anti-mouse IgG and IgM (HRP conjugate, Pierce) at 1: 20 000 for 1 h at room temperature on a rocker platform, with buffer washes between each step. Immunoreactive proteins were visualized with enhanced chemiluminescence (ECL, Pierce, West Pico SuperSubstrate). The following antibodies were used:

**Antibodies directed against cytokeratins**

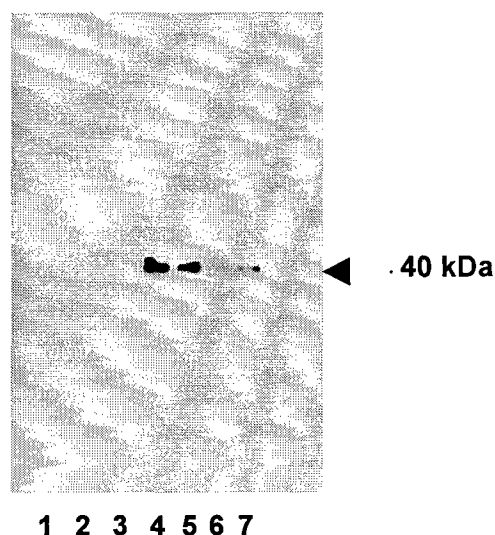
<b>Antibody designation</b>	<b>Company</b>	<b>Working dilution</b>	<b>Antigen Specificity</b>
Clone C-50	NeoMarkers	1 : 100	Cytokeratins 5,8 – simple and stratified epithelia, recognizes proteins of 58 kDa (5) and 52.5 kDa (8).
Clone KS-1A3	Novocastra	1 : 250	Cytokeratin 13-suprabasal marker (nonkeratinizing stratified squamous epithelial differentiation), acidic intermediate filament protein – 54 kDa.
RCK 108	ICN	1 : 100	Cytokeratin 19-simple epithelia as well as basal layer of nonkeratinizing stratified epithelia
Clone AE1/AE3	NeoMarkers	1: 200	AE1- recognizes the 56.5, 50, 50, 48, and 40 kDa keratins of acidic subfamily. AE3- reacts with basic keratins of 65-67, 64, 59, 58, 56, and 52 kDa
LL001	Courtesy of Dr. B. A. Dale	1:200	Cytokeratin 14- distinguishes stratified epithelial cells from simple epithelial cells, acidic subfamily 50 kDa protein.

The results of these preliminary data are represented in Figure 1. All of the cell extracts stained positively with the PAN antibody (Figure 1F), confirming the epithelial origin of the cells and validating our preliminary immunocytochemical staining. Results of staining for individual keratins suggest that the cell culture growth conditions have marked effects on the expression of the keratins tested to date. For example, though phenol red-containing media is typically used for keratinocytes and other similar cell types, growth in this medium produced cells with a completely different keratin phenotype, as compared with growth in media that was otherwise identical except for the absence of phenol red. Growth in phenol red produced pattern most consistent with a simple epithelium and/or the most basal layers of a stratified epithelium. Extracts of these cells stained strongly K19 positive and had almost no staining with MAb directed against K13 or K14. In contrast, the same cells grown in PRF showed a keratin pattern more consistent with a suprabasal or even superficial layer of stratified epithelium. Staining with MAb directed against K13 and K14 was strong, while staining with MAb directed against K19 was nearly absent. Addition of serum produced a keratin expression pattern most suggestive of suprabasal layers in intact stratified epithelium. Similar patterns but with lesser intensity of staining was seen in extracts from cells treated with 17- $\beta$ -estradiol.

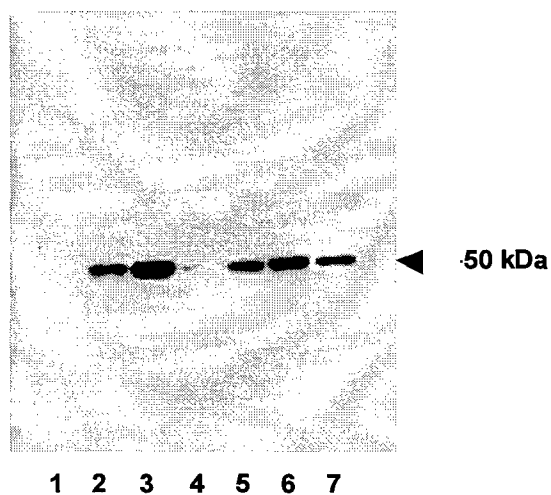
**A**



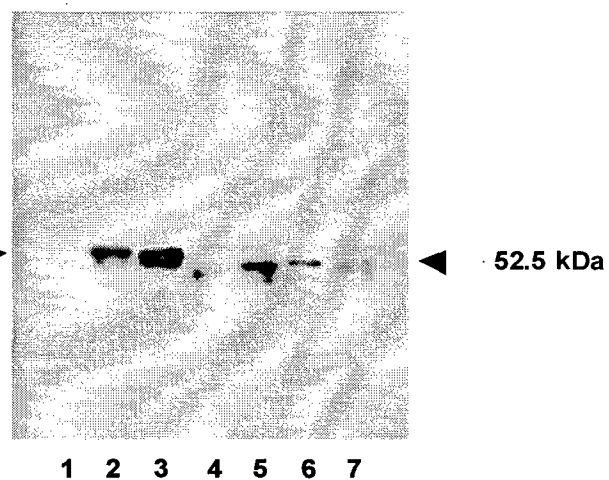
**B**

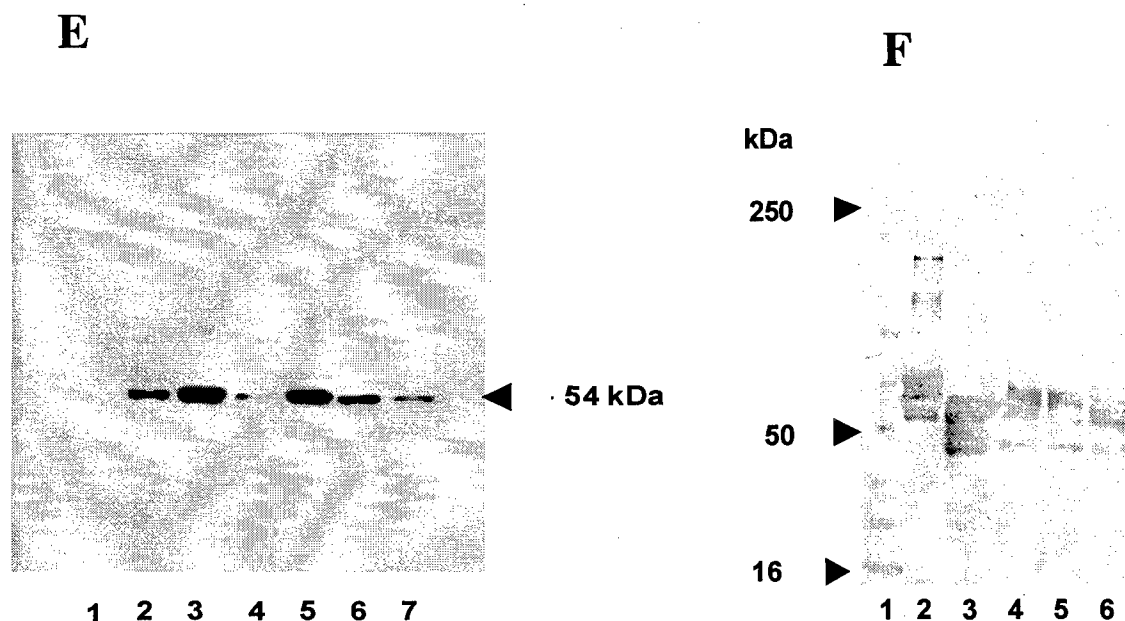


**C**



**D**





**Figure 1. Identification of human vaginal keratins by SDS-PAGE and Western blot analysis.**

**A.** Coomassie Brilliant Blue staining of 8-16% gradient polyacrylamide gel. **B:** K19; **C:** K14; **D:** K8; **E:** K13. Western blots developed with enhanced chemiluminescence method.

**Lane 1** ProSieve Protein Markers.

**Lane 2** Epidermal extract (courtesy of B. Dale).

**Lane 3** Extract of cultured human neural foreskin keratinocytes (courtesy of B. Dale).

**Lane 4** Extract of keratins from vaginal cells (line V26) grown in keratinocyte-SFM media.

**Lane 5** Extract of keratins from vaginal cells (V26) grown in keratinocyte-SFM media and treated with addition of fetal bovine serum for 48 hours prior to harvest.

**Lane 6** Extract of keratins from vaginal cells (V26) grown in phenol red-free- (PRF)-SFM media.

**Lane 7** Extract of keratins from vaginal cells (V26) grown in PRF-SFM media and stimulated with 250 pg/ml 17- $\beta$ -estradiol for 24 hours.

**Lane 8** SeeBlue pre-stained standards.

**F:** PAN; Western blot developed with a colorimetric alkaline phosphatase method

**Lane 1** ProSieve Protein Markers.

**Lane 2** Epidermal extract (courtesy of B. Dale).

**Lane 3** Extract of keratins from vaginal cells (line V26) grown in keratinocyte-SFM media.

**Lane 4** Extract of keratins from vaginal cells (V26) grown in keratinocyte-SFM media and treated with addition of fetal bovine serum for 48 hours prior to harvest.

**Lane 5** Extract of keratins from vaginal cells (V26) grown in phenol red-free- (PRF)-SFM media.

**Lane 6** Extract of keratins from vaginal cells (V26) grown in PRF-SFM media and stimulated with 250 pg/ml 17- $\beta$ -estradiol for 24 hours.

**Lane 7** SeeBlue pre-stained standards.



### 3. Recommendations in relation to the Statement of Work

1. Primary bladder epithelial cells: We will continue to collaborate with Drs. Atala and Grady to cultivate bladder cells, establishing additional frozen stocks and cell pellets for purification as needed.
2. Primary vaginal epithelial cells: In the next funding year, we will complete a few experiments to characterize VECs and complete a manuscript in preparation, describing these data. We have recently performed Western blots using antibodies directed against K6, K10, and K18 (not shown). The results of these experiments will be integrated with the data shown above in the manuscript.

#### b. Task 2, Months 7 to 12: extraction and purification of GSLs from bladder and vaginal cell cultures

##### 1. Experimental methods, assumptions and procedure

A total of 10 bladder epithelial cell samples have been extracted and partially purified to date. The methods used to extract GSLs from primary cultures of human bladder epithelial cells and VECs as needed were unchanged from those described in our preliminary data: at each step, GSLs were chromatographed in various organic solvent systems and stained with orcinol (carbohydrate detection stain) to assess the purity of individual bands. In addition, HPTLC bacterial overlay procedures as described below were performed to identify and monitor the purification of GSLs of interest. Briefly, the GSL isolation and purification steps are as follows: cell cultures were maintained as described (13) then cells were trypsinized, pelleted, and washed, and the total GSLs were obtained by extracting the pellets with 10 volumes isopropanol:hexane:water (IHW; 55:25:20 by volume) with sonication in a warm bath and centrifugation at 2,500 RPM for 10 minutes. Samples were subjected to a modified Folch procedure to obtain the upper and lower phase GSLs (15), free salts and sugars removed with dialysis and/or reverse phase chromatography, and then contaminating phospholipids were removed using acetylation/deacetylation procedures. The packed cell volume of the largest pellet processed is 6 ml.

##### 2. Results and discussion

Figure 2 (Appendix) shows a summary of the key cell pellets subjected to the procedures shown above. The samples have been extracted as described above and subjected to HPTLC and bacterial overlay assay with E. coli R45 to reveal globoseries GSLs.

### 3. Recommendations in relation to the Statement of Work

This stage of the GSL characterization is completed with respect to bladder epithelial cells. We will perform further extraction procedures with vaginal epithelial cells as needed.

#### c. Task 3, Months 7 to 12: bacterial overlay assays

##### 1. Experimental methods, assumptions and procedure

This assay involves separating GSLs on HPTLC plates and overlaying the plates with radiolabeled bacteria (3, 18). E. coli organisms were metabolically labeled with [<sup>35</sup>S]methionine as previously described (3). S. saprophyticus organisms were also metabolically labeled with [<sup>35</sup>S]methionine using a gonococcal labeling method (Mandrell, unpublished data) that we adapted for S. saprophyticus (Stapleton et al., manuscript in preparation). The organisms have a final specific activity of 0.01 cpm/organism, similar to the specific activity achieved using comparable methods for E. coli (3). Radioactive bacterial overlay assays were performed as previously described, with a HPTLC plate run in parallel for orcinol staining. For the bacterial overlay assays with E. coli, we have continued to primarily use metabolically labeled wild

type E. coli R45 (3), which expresses the pap-encoded class II adhesin (19) and thus specifically recognizes globoseries GSLs.

## **2. Results and discussion**

### GSL expression in cultured vaginal epithelial cells

Data described in last year's report have been confirmed and are being incorporated into a manuscript in preparation.

### GSL expression in cultured bladder epithelial cells

Data from experiments investigating the expression of GSLs that bind either E. coli or S. saprophyticus are being incorporated into two manuscripts in preparation.

## **3. Recommendations in relation to the Statement of Work**

Manuscripts will be completed; experiments will be repeated only as needed.

### **d. Task 4, Months 7 to 12: immunostaining assays**

#### **1. Experimental methods, assumptions and procedure**

Working with Dr. Hakomori, we attempted to harvest additional amounts of Mab ID4 from hybridoma cultures, using standard techniques. These are for use in immunostaining experiments using standard techniques, referenced in our earlier work [Stapleton, 1992 #40].

#### **2. Results and discussion**

After a considerable effort in concert with Dr. Hakomori's lab, because of a labeling error, we found that we had harvested an antibody with a similar name, directed against Le<sub>y</sub>.

#### **3. Recommendations in relation to the Statement of Work**

1. We have made plans with Dr. Hakomori to again try to harvest Mab ID4 from the appropriate hybridoma cultures.
2. Additional immunostaining assays will be performed as needed to confirm presumptive identification of GSLs in assay described above.

### **e. Task 5, Months 13 to 24: carbohydrate structural analysis; and**

### **f. Task 6, Months 25 to 36: data analysis and publication**

#### **1. Experimental methods, assumptions and procedure**

a. Definitive carbohydrate structural analysis: Full details of the methods described in previous reports for purifying and performing definitive carbohydrate structural analysis of compounds mentioned below are referenced in Dr. Stroud's publication. Methods newly adopted are described below.

b. New methods using exoglycosidases: I last year's report, we described pilot studies of using a new method of deducing carbohydrate structures among mixtures. The principle behind this method is as follows: when we are working with a mixture of structures purified from a source such as VECs, often we have tools for definitively identifying some of the bands seen when the mixture is separated on HPTLC,

such as reactivity with specific MAbs directed against that carbohydrate structure. In addition, some of the structures of interest are members of families of GSLs, such as the globoseries, which differ from one another in structure only by one or two terminal sugar(s). Thus, we can identify some compounds for which we have specific MAbs by cleaving the relevant terminal sugar(s) with specific exoglycosidases and then using staining with MAbs, bacterial overlay assays, and assessment of relative mobilities on HPTLC to deduce the structures(s). An example of how we can take advantage of these structural relationships is shown below in Table 1. We piloted this approach for identifying the globoseries GSLs that bind *pap*-adhesin expressing *E. coli* in our bladder cell extracts. Using Gb5 synthetically produced by Dr. Stroud we used specific exoglycosidases to first cleave the terminal galactose to obtain Gb4 (globoside), followed by another enzyme to cleave terminal *N*-acetylgalactosamine from Gb4 to obtain Gb3, an epitope for which we have a specific MAb.

**Table 1. Glycosphingolipid structures**

<b>Globoseries GSLs</b>	<b>Structure core: Gal<math>\alpha</math>1-4 Gal<math>\beta</math>1-R</b>
CTH (Gb3)	Gal $\alpha$ 1-4 Gal $\beta$ 1-4 Glc $\beta$ 1-1cer
Globoside (Gb4)	GalNAc $\beta$ 1-3 Gal $\alpha$ 1-4 Gal $\beta$ 1-4 Glc $\beta$ 1-1cer
Galactosyl globoside (Gb5)	Gal $\beta$ 1-3GalNAc $\beta$ 1-3 Gal $\alpha$ 1-4 Gal $\beta$ 1-4 Glc $\beta$ 1-1cer

## 2. Results and discussion

Expansion of our studies with exoglycosidase treatments using purified globoseries compounds proved more expensive and less efficacious than anticipated when applied to mixtures of materials. Thus, to investigate the chemistry of the glycolipids found in cultured cells of human primary bladder cell lines, a sensitive mass spectrometric technique was developed for native GSLs found in complex mixtures. Various purified neutral and monosialylated GSLs were examined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) in the reflector mode. Native globo-series GSLs, Gb<sub>4</sub> and Gb<sub>5</sub>, and the monosialyl ganglioside GM<sub>1</sub> were found to give good spectra in the positive ion mode (detection limits of 10-100 pmol; see Appendix).

MALDI-TOF mass spectrometric analysis of a total glycolipid fraction isolated from a human primary bladder cell line also gave a good spectrum of glycolipid derived molecular ions (see Appendix). Post source decay experiments are underway on these mass-selected ions to obtain further structural information.

## 3. Recommendations in relation to the Statement of Work

1. As needed, we will continue MALDI-TOF experiments and work with Dr. Levery to perform carbohydrate structural analysis as appropriate.

### 2. Technical Objective 2

a. Task 1, Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures

#### 1. Experimental methods, assumptions and procedure

Methods described in our preliminary data and prior reports.

#### 2. Results and discussion

(contains unpublished and/or proprietary information)

### **3. Recommendations in relation to the Statement of Work**

These data are completed and are incorporated in prior reports and in manuscripts in preparation.

#### **b. Task 2, Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs**

##### **1. Experimental methods, assumptions and procedure**

Methods used were as described in our preliminary data.

##### **2. Results and discussion; and 3. Recommendations in relation to the Statement of Work**

These data are completed and are incorporated in prior reports and in manuscripts in preparation.

#### **c. Task 3, Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays**

##### **1. Experimental methods, assumptions and procedure**

Methods used were as described in our prior reports and preliminary data.

##### **2. Results and discussion; and**

##### **3. Recommendations in relation to the Statement of Work**

These data are completed and are incorporated in prior reports and in manuscripts in preparation.

### **3. Technical Objective 3**

#### **a. Task 1, Months 24 to 42: synthesis of linear mimetic compounds**

##### **1. Experimental methods, assumptions and procedure; and**

##### **2. Results and discussion**

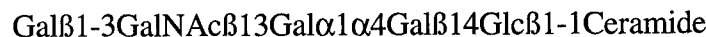
As noted in last year's report, we pursued a slightly different approach to generate the parent compounds for synthesis of the linear mimetics, namely enzymatic synthesis of the parent GSLs. Details of the progress in this area are as follows:

##### Enzymatic synthesis of globoseries based GSL receptor analogs

An underlying goal of the experiments described in this report is to chemically modify sialosyl galactosyl globoside (SGG) to identify the functional groups that give rise to its high affinity characteristics as a receptor for uropathogenic *E. coli*. To generate relatively large quantities of SGG for our chemical modification studies, we extracted chicken pectoral muscle GSLs, separated them on HPTLC as described above, and performed bacterial overlay assays with representative *E. coli* strains described above. We detected an *E. coli*-binding GSL that co-migrates with SGG in ganglioside fractions of chicken muscle (data not shown).

We have also continued to pursue the partial enzymatic synthesis of galactosyl globoside (Gb<sub>3</sub>), the immediate precursor to SGG. We detected relatively high levels of  $\beta$ 1-3 galactosyltransferase activity capable of catalyzing the biosynthesis of Gb<sub>3</sub> using globoside (Gb<sub>4</sub>) as an acceptor substrate in two cell lines. While pursuing these strategies a recently cloned  $\beta$ 1 $\rightarrow$ 3Galactosyltransferase was obtained from

Dr. Henrik Clausen at the University of Copenhagen and assayed using Gb<sub>4</sub> as an acceptor substrate. The enzyme efficiently converted Gb<sub>4</sub> in a reaction mixture containing UDP-galactose and MnCl<sub>2</sub> to a slower migrating glycolipid that co-migrated with a Gb<sub>5</sub> standard when assessed by thin-layer chromatography. The enzyme was expressed as a soluble construct in insect cells (baculovirus system) purified to homogeneity and used to biosynthesize the putative Gb<sub>5</sub>. A large-scale purification of Gb<sub>4</sub> from human erythrocytes was completed and used as the acceptor substrate. Gb<sub>5</sub> was synthesized, purified and sent to Dr. Steve Levery at the University of Georgia for structural analysis. The structure of Gb<sub>5</sub> was confirmed by <sup>1</sup>H-NMR as follows:



Since a sufficient amount of Gb<sub>5</sub> has now been synthesized, we will sialylate the terminal galactose of Gb<sub>5</sub> using CMP-sialic acid as the donor substrate and a commercially available  $\alpha 2,3$  sialyltransferase to generate SGG for further studies.

In parallel with these studies a rat liver  $\alpha 1 \rightarrow 2$  fucosyltransferase previously thought to only utilize the ganglioside GM<sub>1</sub> as an acceptor substrate was assayed using Gb<sub>5</sub> as a substrate. Preliminary data suggests that this enzyme actually has a lower K<sub>m</sub> for Gb<sub>5</sub> than GM<sub>1</sub> and catalyzes the biosynthesis of Globo-H. The enzyme was expressed in COS-7 cells and a detergent extract of the cells was used as the enzyme source in a reaction mixture containing GDP-fucose, MnCl<sub>2</sub> and the detergent Triton CF-54. The final product has been confirmed by NMR. Although this  $\alpha 1 \rightarrow 2$  fucosyltransferase was identified and characterized over 15 years ago the substrate Gb<sub>5</sub> was not easily available until now. Sufficient quantities of SGG and globo-H will soon be available and we will have a complete panel of globo-series glycolipids to perform additional *E. coli* binding studies.

#### Cloning of human $1 \rightarrow 4$ Galactosyltransferases

The Gal $\alpha 1 \rightarrow 4$ Gal sequence found in all globo-series glycolipids is the minimum structural element required for binding of uropathogenic strains of *E. coli* expressing a Class II *pap*-encoded adhesin. This structure also forms the basis for the human P histo-blood group system. The gene encoding the glycosyltransferase responsible for catalyzing the synthesis of this linkage has now been identified. The sequence of a recently cloned  $\alpha 4$ GlcNAc-transferase was used in a BLAST search to identify a novel homologous gene. Expression of full coding constructs of the gene, designated  $\alpha 4$ Gal-T1, in insect cells revealed P<sub>k</sub> synthase activity, but no P<sup>1</sup> activity (see Appendix). The LgtC gene product from *N. gonorrhoea* is involved in the biosynthesis of lipidoligosaccharide (LOS) and encodes an  $\alpha 1 \rightarrow 4$ Galactosyltransferase. Expressed sequence tags (ESTs) based on sequence similarity to the LgtC gene product were retrieved using the tBLASTn algorithm against a human EST database. Blast analysis of gene databases resulted in identification of two human genes encoding putative type II transmembrane proteins. The genes have open reading frames encoding 349 and 371 amino acid residues. To date, expression of full coding constructs of each gene in insect cells failed to demonstrate  $\alpha 4$ Gal-T activity.

### **3. Recommendations in relation to the Statement of Work**

1. SGG synthesis: We now have sufficient amounts of Gb<sub>5</sub> are synthesized to sialylate the terminal galactose of Gb<sub>5</sub> using CMP-sialic acid as the donor substrate and a commercially available  $\alpha 2,3$  sialyltransferase to generate SGG for further studies. The final product will be confirmed by <sup>1</sup>H-NMR by Dr. Levery.

## 7. KEY RESEARCH ACCOMPLISHMENTS

- Establishment of in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells
- Characterization of glycosphingolipids (GSLs) expressed by these epithelial cells
- Characterization of bacterial adherence to these cells by organisms that are key uropathogens for healthy young women
- Establishment of the principle that GSLs are essential for the adherence of pap-adhesin-expressing uropathogenic E. coli in these systems
- Studies of the effects of exogenous estrogen on the expression of GSLs and keratins in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on bacterial adherence
- Cloning of a human  $\alpha$ 1-4Galactosyltransferase
- Enzymatic synthesis of globoseries based GSL receptors
- Obtaining interpretable MALDI spectra on a complex mixture of native GSLs in small quantities, not requiring prior permethylation

## 8. REPORTABLE OUTCOMES

### a. Manuscripts, abstracts and presentations

#### Manuscripts

1. Schlager TA, Whittam TS, Hendley JO, Wilson RA, Bhang JL, Grady R, Stapleton AE. Expression of virulence factors among Escherichia coli isolated from the periurethra and urine of children with neurogenic bladder on intermittent catheterization. *Pedi Infect Dis J* 2000;19:37-41.
2. Hooton TM, Stapleton AE, Roberts PL, Winter C, Scholes D, Bavendam T, Stamm WE. Perineal anatomy and urine voiding characteristics in young women with and without recurrent urinary tract infections. *Clin Infect Dis* 1999;29:1600-1601.
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8. Steffensen R, Carlier K, Wiels J, Levery S.B, Stroud M, Cedergren B, Nilsson Sojka B, Bennett EP, Jersild C, Clausen, H. Cloning and expression of the histo-blood group Pk UDP-galactose: Galbeta-4G1cbeta1-cer alpha1, 4-galactosyltransferase. Molecular genetic basis of the p phenotype. *J Biol Chem* 2000;275:16723-16729.

#### Abstracts

1. Carriage of Escherichia coli and expression of virulence factors in urine and periurethra of patients with neurogenic bladder on intermittent catheterization. International Bladder Symposium, November 4-7, 1999, Washington DC

#### Manuscripts in press

1. Molecular strategies for the prevention and treatment of urinary tract infections. *Molecular Medicine Today*.

#### Presentations and Meetings

1. Moderator and Scientific Committee, 2001 International Bladder Symposium
2. Invited speaker, University of Washington Science in Medicine New Investigator series, 1999-2000 (also received award in connection with this lecture series)
3. Obstetrics and Gynecology Grand Rounds, 2000

#### **Development of cell lines**

Primary cultures of vaginal epithelial cells derived from individuals have been developed for the purposes of these studies. However, these cells have not been provided to other investigators as a "reagent" and are not suitable for an application of that nature, such as submission to a cell banking facility.

#### **9. CONCLUSIONS**

Our initial years of funding were focused on refining the techniques we developed for culturing primary vaginal epithelial cells and on adapting Dr. Atala's methods for culturing primary human urothelial cells to our model system. We have now established these in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells for the identification, purification and structural characterization of E. coli and S. saprophyticus-binding glycosphingolipid moieties. We have completed studies characterizing bacterial adherence to these cells by organisms that are key uropathogens for healthy young women, E. coli and S. saprophyticus. We have also established that GSLs are essential for the adherence of pap-adhesin-expressing uropathogenic E. coli in these systems. We have completed the first phase of studies of the effects of exogenous estrogen on the expression of GSLs in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on GSL and keratin expression and bacterial adherence. We have established and now developed several new collaborations that will allow us to expand and advance our studies related to epithelial biology, especially those of the vaginal epithelium. Establishing our model of the vaginal epithelium opens opportunities for studying numerous aspects of women's urogenital health, including testing vaginal products, probiotics, and contraceptives;

understanding protective roles of organisms in the normal flora; and studying the cellular effects of hormone replacement therapy on a key target tissue, the vagina.

The globoseries GLSs have not been thoroughly studied in normal adult tissues. Thus, the new approaches taken by Dr. Stroud in undertaking the enzymatic synthesis of globoseries GSLs and cloning of a human  $\alpha$ 1-4Galactosyltransferase have yielded basic findings that are important in the field of glycobiology, apart from their relevance to this work.

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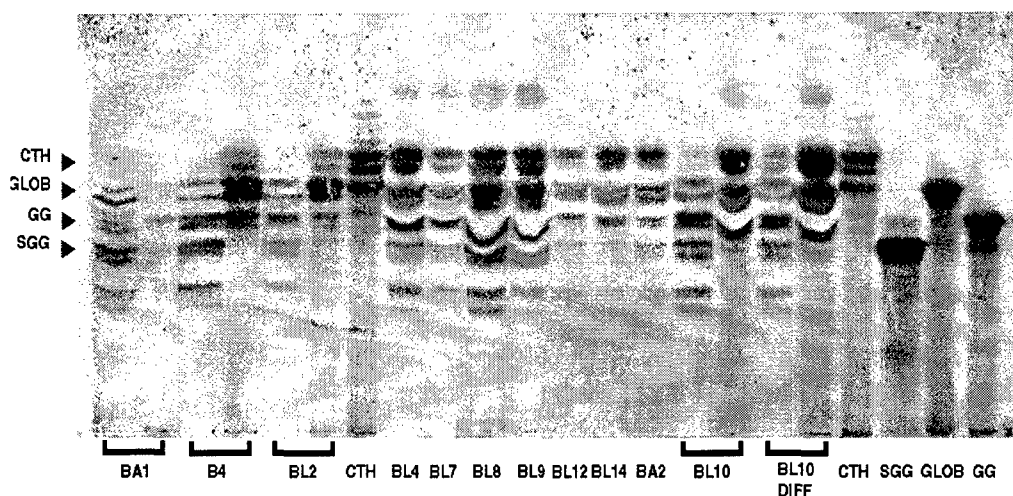
## 11. APPENDICES

**Appendix A:** reprint of Stroud MR, Stapleton AE, Lavery SB. The P histo-blood group related glycosphingolipid sialosylgalactosylgloboside as a preferred binding receptor for uropathogenic Escherichia coli: Isolation and structural characterization from human kidney. Biochemistry 1998;37:17420-17428

**Appendix B:** MALDI-TOF spectra for Gb<sub>4</sub>, Gb<sub>5</sub>, GM-1, and the complex mixture of GSLs described in the text.

**Appendix C:** reprint of Steffensen R, Carlier K, Wiels J, Lavery S.B, Stroud M, Cedergren B, Nilsson Sojka B, Bennett EP, Jersild C, Clausen, H. Cloning and expression of the histo-blood group Pk UDP-galactose: Galbeta-4Glcbeta1-cer alpha1, 4-galactosyltransferase. Molecular genetic basis of the p phenotype. J Biol Chem 2000;275:16723-16729.

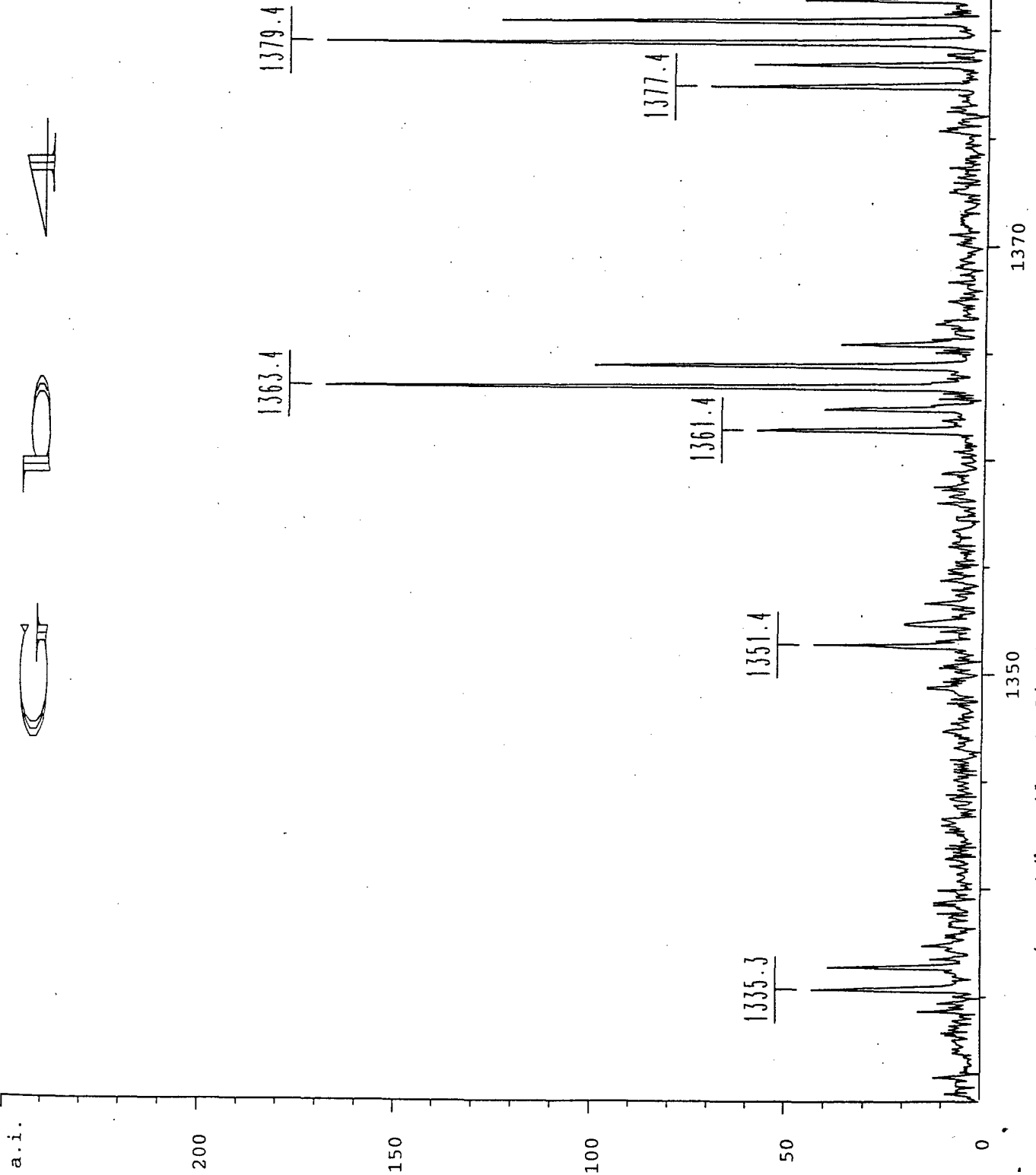
## APPENDIX A



**Figure 2. Extraction of glycosphingolipids from primary bladder epithelial cells.** As described in the text, primary bladder epithelial cells were grown to near confluence, harvested by scraping, and subjected to GSL isolation procedures. Partially purified GSLs were then separated on HPTLC and overlaid with  $^{35}$ -S metabolically labeled *E. coli* R45 to reveal globoseries GSLs. CTH (ceramide trihexosyl; globotriaosyl ceramide; GLOB, globoside, globotetraosylceramide); SGG (sialosyl galactosyl globoside); and GG (galactosyl globoside) are GSL standards.

**Appendix B:** MALDI-TOF spectra for Gb<sub>4</sub>, Gb<sub>5</sub>, GM-1, and the complex mixture of GSLs described in the text.

10  $\mu$ g stock  
500 ng



a.i.

500

400

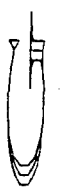
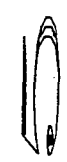
300

200

100

0

10% stock  
500 ng  
di-



1525.6

1523.6

1497.5

1495.5

1539.6

1490

1510

1530

m/z

/export/home/data/yuliya/Gb5\_10mkg/lRef/pdata/1 unknown Wed Mar 1 13:52:13 2000

a.i.

2500

2000

1500

1000

500



"

1574.0

1545.9

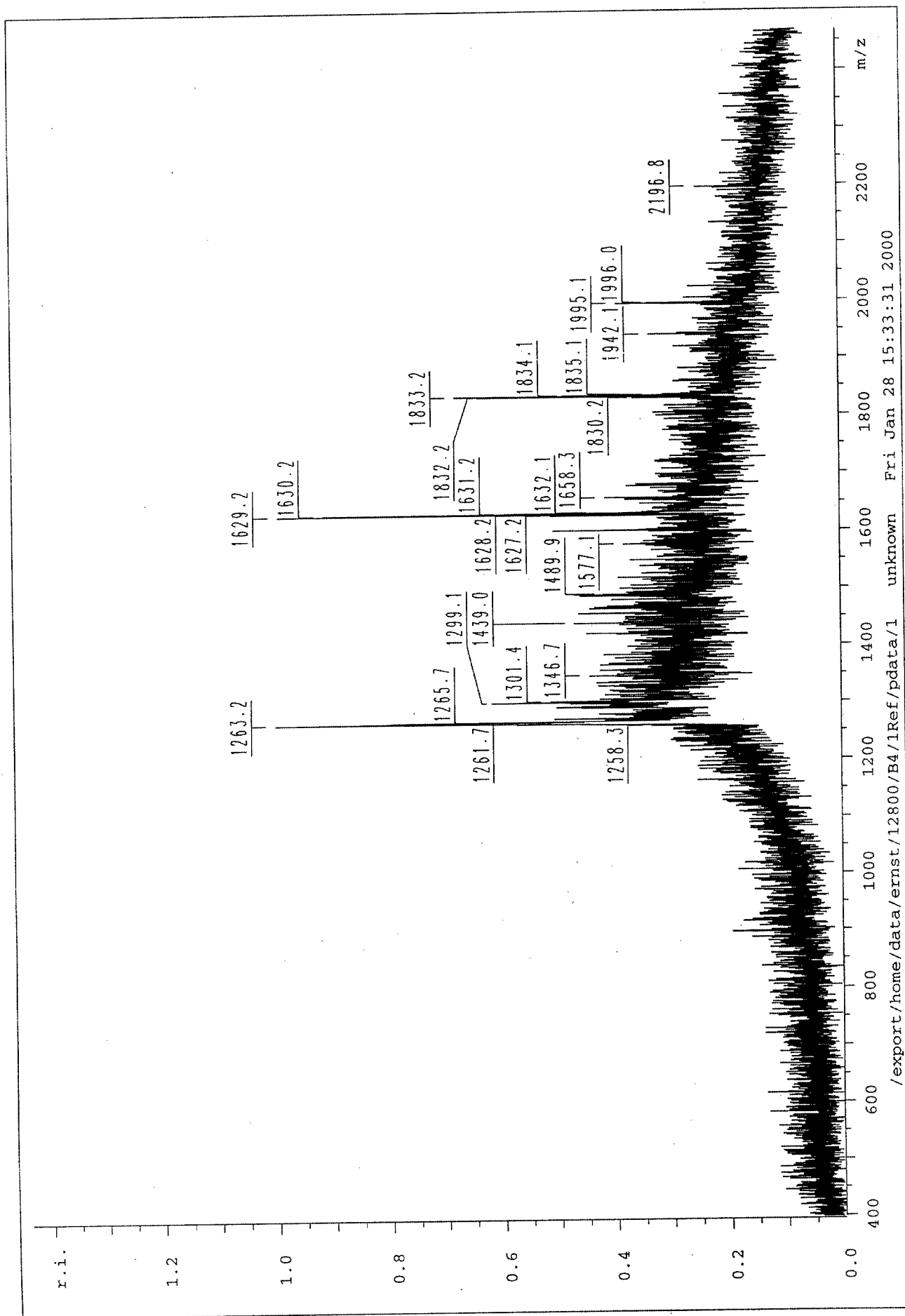
1540

1560

1580

m/z

/export/home/data/yuliya/Gm1\_10mkg/1Ref/pdata/1 unknown Wed Mar 1 13:33:03 2000



# Cloning and Expression of the Histo-blood Group P<sup>k</sup> UDP-galactose: Gal $\beta$ 1-4Glc $\beta$ 1-Cer $\alpha$ 1,4-Galactosyltransferase

MOLECULAR GENETIC BASIS OF THE p PHENOTYPE\*

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The molecular genetic basis of the P histo-blood group system has eluded characterization despite extensive studies of the biosynthesis of the P<sub>1</sub>, P, and P<sup>k</sup> glycolipids. The main controversy has been whether a single or two distinct UDP-Gal:Gal $\beta$ 1-R 4- $\alpha$ -galactosyltransferases catalyze the syntheses of the structurally related P<sub>1</sub> and P<sup>k</sup> antigens. The P<sub>1</sub> polymorphism is linked to 22q11.3-ter. Data base searches with the coding region of an  $\alpha$ 4GlcNAc-transferase identified a novel homologous gene at 22q13.2 designated  $\alpha$ 4Gal-T1. Expression of full coding constructs of  $\alpha$ 4Gal-T1 in insect cells revealed it encoded P<sup>k</sup> but not P<sub>1</sub> synthase activity. Northern analysis showed expression of the transcript correlating with P<sup>k</sup> synthase activity and antigen expression in human B cell lines. Transfection of P<sup>k</sup>-negative Namalwa cells with  $\alpha$ 4Gal-T1 resulted in strong P<sup>k</sup> expression. A single homozygous missense mutation, M183K, was found in six Swedish individuals of the rare p phenotype, confirming that  $\alpha$ 4Gal-T1 represented the P<sup>k</sup> gene. Sequence analysis of the coding region of  $\alpha$ 4Gal-T1 in P<sub>1</sub>+/- individuals did not reveal polymorphisms correlating with P<sub>1</sub>P<sub>2</sub> typing.

The P histo-blood group system is the last of the known carbohydrate defined blood group systems for which the molecular genetic basis has not yet been clarified. The P blood group system involves two major blood group phenotypes, P<sub>1</sub>+ and P<sub>1</sub>-, with approximate frequencies of 80% and 20%, respectively (1, 2). P<sub>1</sub>- individuals normally express the P antigen (P<sub>1</sub>- is designated P<sub>2</sub> when P antigen expression is demonstrated), but the rare P<sup>k</sup> phenotype lacks the P antigen, while

the rare p phenotype lack both P and P<sup>k</sup> antigens (for reviews, see Refs. 3–7). The P<sub>1</sub>+ phenotype is defined by expression of the neolacto-series glycosphingolipid P<sub>1</sub> (for structures, see Table I) (8). In contrast, the P, P<sup>k</sup>, and p antigens constitute intermediate steps in biosynthesis of globo-series glycolipids and give rise to P<sub>1</sub><sup>k</sup>, P<sub>2</sub><sup>k</sup>, and p phenotypes (9). Although the rare P<sup>k</sup> phenotype shows the same frequency of P<sub>1</sub> antigen expression as individuals expressing the P antigen, the p phenotype is always associated with lack of P<sub>1</sub> antigen expression. Extensive studies of the chemistry, biosynthesis, and genetics of the P blood group system identified the antigens as being exclusively found on glycolipids, with the blood group specificity being synthesized by at least two distinct glycosyltransferase activities; UDP-galactose: $\beta$ -D-galactosyl- $\beta$ 1-R 4- $\alpha$ -D-galactosyltransferase ( $\alpha$ 4Gal-T)<sup>1</sup> activity(ies) for P<sup>k</sup> and P<sub>1</sub> syntheses and UDP-GalNAc:Gb<sub>3</sub> 3- $\beta$ -N-acetylgalactosaminyltransferase activity (EC 2.4.1.79) for P synthesis (for reviews, see Refs. 6 and 7). At least two independent gene loci, P and P<sub>1</sub>P<sup>k</sup>, are involved in defining these antigens. The P blood group-associated LKE antigen, shown to be the extended sialylated Gal-globoside structure (10), may involve polymorphism in an  $\alpha$ 2,3-sialyltransferase activity.

A long-standing controversy has been whether a single or two independent  $\alpha$ 1,4-galactosyltransferases catalyze the synthesis of the P<sub>1</sub> neolacto-series glycolipid antigen and the P<sup>k</sup> globo-series structure (3–7). Several hypotheses have been proposed, including: (i) a model with two distinct functional genes being allelic or non-allelic, where the P<sub>1</sub> gene encodes a broadly active  $\alpha$ 4Gal-T, the P<sup>k</sup> gene encodes a restricted  $\alpha$ 4Gal-T, and a null allele encodes a non-functional protein; (ii) a model with two distinct non-allelic genes, where P<sub>1</sub> encodes an  $\alpha$ 4Gal-T that can only synthesize P<sub>1</sub> structures and the P<sup>k</sup> encodes an  $\alpha$ 4Gal-T that only synthesize the P<sup>k</sup> structure; and (iii) a model where one gene locus encodes an  $\alpha$ 4Gal-T that is modulated by an independent polymorphic gene product to synthesize both P<sub>1</sub> and P<sup>k</sup> structures. Bailly *et al.* (11) reported that kidney microsomal  $\alpha$ 4Gal-T activity from P<sub>1</sub> individuals does not compete for the two substrates used by P<sub>1</sub> and P<sup>k</sup>  $\alpha$ 4Gal-T activities, and no accumulative effect in P<sub>1</sub> synthase activity was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ245581.

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<sup>1</sup> The abbreviations used are:  $\alpha$ 4Gal-T, UDP-galactose: $\beta$ -D-galactose-R 4- $\alpha$ -D-galactosyltransferase; UTR, untranslated region; EST, expressed sequence tag; PCR, polymerase chain reaction; bp, base pair(s); nt, nucleotide(s); EBV, Epstein-Barr virus; CDH, ceramide dihexoside; CTH, ceramide trihexoside; Gb<sub>3</sub>, globotriaosylceramide.



TABLE I  
Structures of glycosphingolipids referred to in this study

CDH, ceramide dihexoside (LacCer, lactosylceramide); CTH, ceramide trihexoside (Gb<sub>3</sub>, globotriaosylceramide); globoside, Gb<sub>4</sub> (globotetraosylceramide); Cer, ceramide; Gal, D-galactose; Glc, D-glucose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; NeuAc, N-acetyl-neuraminic acid.

	Structure	P blood group antigen
CDH, LacCer	Galβ1-4Glcβ1-1Cer	P
CTH, Gb <sub>3</sub>	Galα1-4Galβ1-4Glcβ1-1Cer	P <sup>k</sup>
Globoside	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	P
Sialyl-Gal-globoside	NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	LKE
Paragloboside, PG	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	
P <sub>1</sub>	Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	P <sub>1</sub>

observed when mixing microsomal fractions from individuals of P<sub>1</sub> and P<sup>k</sup> groups. Based on this, Bailly and colleagues suggested the existence of two distinct genes, coding for one P<sub>1</sub>  $\alpha 4$ Gal-T with exclusive activity for neolacto-series substrates and one P<sup>k</sup>  $\alpha 4$ Gal-T with exclusive activity for the globo-series substrate. Since p individuals lack the P<sub>1</sub> antigen, this model inferred that two independent genetic events inactivating both genes was responsible for the p phenotype.

Several approaches to gain insight into the P blood group  $\alpha 4$ Gal-T gene(s) have been attempted. Purification of the mammalian enzymes has not been successful, but identification and cloning of a bacterial  $\alpha 4$ Gal-T involved in lipopolysaccharide biosynthesis (12, 13) potentially provided a strategy to clone the mammalian genes using sequence similarity. Previously, a bacterial  $\alpha 3$ -fucosyltransferase was identified in *Helicobacter pylori* using a short sequence motif conserved among mammalian  $\alpha 3$ -fucosyltransferases (14). BLAST analysis of gene data bases with the coding region of the  $\alpha 4$ Gal-T gene from *Neisseria meningococcae* resulted in identification of two human genes encoding putative type II transmembrane proteins with low sequence similarity to the bacterial gene.<sup>2</sup> The genes have open reading frames encoding 349 (EST cluster Hs.251809) and 371 (EST cluster Hs.82837) amino acid residues and are located at 8q24 and 3p21.1, respectively. Previously, we established Epstein-Barr virus (EBV)-transformed B cells from two p individuals (15). Only the gene at 3p21.1 was found to be expressed in the EBV-transformed p cells, as well as in Ramos cells known to have high P<sup>k</sup>  $\alpha 4$ Gal-T activity. Sequencing of the coding region of the gene showed no mutations in p cells. Finally, expression of full coding or truncated, secreted constructs of either gene in insect cells failed to demonstrate glycosyltransferase activity with a large panel of substrates, including lactosylceramide, for P<sup>k</sup>  $\alpha 4$ Gal-T activity.

The P<sub>1</sub> polymorphism is linked to 22q11.3-ter (16), whereas no information is available for the p phenotype. In the present report, we used BLAST searches of gene data bases with the coding region of a recently cloned human  $\alpha 4$ GlcNAc-transferase located at 3p14.3 (17) to identify a novel homologous gene located at 22q13.2. Expression of full coding constructs of the gene, designated  $\alpha 4$ Gal-T1, in insect cells revealed P<sup>k</sup> synthase activity, but no P<sub>1</sub> activity was demonstrated. Sequence analysis of the coding region of  $\alpha 4$ Gal-T1 in P<sub>1</sub> and P<sub>2</sub> individuals did not reveal polymorphisms correlating with the P<sub>1</sub>P<sub>2</sub> blood group phenotypes. In contrast, a single homozygous missense mutation, M183K, was found in six Swedish individuals of the rare p type.

#### EXPERIMENTAL PROCEDURES

**Identification and Cloning of  $\alpha 4$ Gal-T1**—tBLASTn analysis of the human genome survey sequences, unfinished high throughput genomic sequences, and dbEST data bases at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD) with

the coding sequence of a human  $\alpha 4$ GlcNAc-transferase recently cloned by Nakayama *et al.* (17), produced a novel open reading frame of 1059 bp with significant similarity. The full coding sequence was available from BAC clone SC22CB-33B7 on chromosome 22 (GenBank™ accession no. Z82176) in a single exon. With the release of the sequence of chromosome 22, the mapping data are cB33B7.1 at 2.65055 × 10<sup>7</sup> to 2.65044 × 10<sup>7</sup> flanked by diaphorase (NADH) and an unknown protein. Linkage analysis of the P<sub>1</sub> polymorphism was originally performed with NADH-cytochrome b<sub>5</sub> reductase (16). Few ESTs cover the coding region (e.g. R45869), but the 3'-UTR is covered by EST Unigene cluster Hs.105956. Available ESTs are mainly derived from tonsil, prostate, and germ cell tumors.

**Identification of Sequence Polymorphisms in the Coding Region of  $\alpha 4$ Gal-T1**—The sequence analysis was performed in three steps. Initially, the coding region of  $\alpha 4$ Gal-T1 from seven P<sub>1</sub>+, five P<sub>1</sub>−, and six p phenotype individuals were sequenced in full by direct sequencing of a genomic fragment of 1295 bp derived by PCR with primer pair HCRS122 (5'-CCAGCCTGGCTCTGGCTGATG) and HCRS126 (5'-CCCGGTGGCAGCTCGGGCCTC) located downstream and upstream of the translational start and stop sites, respectively. The PCR products were sequenced in both directions using the primers HCRS122, HCRS126, HCRS1 (5'-ATCTCACTTCTGAGCTGC), and HCRS4 (5'-GTTGTAGTGGTCCACGAAGTC). Subsequently, the products from two individuals (194 and 321) homozygous for G109 and two (183 and 300) homozygous for A109, randomly selected were subjected to cloning into pBluescript KS+ (Stratagene) followed by sequencing of clones. Finally, a genotyping assay based on *RcaI* restriction enzyme digestion of a PCR product was developed for the identified A109G missense mutation allele. PCR was performed using primer pair HCRS133 (5'-AAGCTCCTGGTCTGATCTGG) and HCRS6 (5'-ACCGAGCACATGAACAGGAAGTT) (30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s), and a total of 31 P<sub>1</sub>+ and 51 P<sub>1</sub>− phenotyped individuals was typed. *RcaI* digestion cleaves the expected product (319 bp) of A109 in two fragments of 182 and 137 bp. The *RcaI* digestion of PCR products was confirmed by Southern analysis on 3 P<sub>1</sub>+ and 2 P<sub>1</sub>− individuals.

**Expression of  $\alpha 4$ Gal-T1 in Insect Cells**—Full coding constructs were prepared by genomic PCR using primer pair HCRS131 (5'-ACCATGTC-CAGCCCCCGACCTC) and HCRS125 (5'-CATGAAATGTACTTGTGAGGGG) and genomic DNA from phenotyped individuals with phenotypes P<sub>1</sub>+ (165) and p (4) (see Table II for sequence). Three different full coding constructs were selected for expression: 67 (A109, T548, G903, G987), 45 (G109, T548, G903, A987), and p5 (A109, A548, G903, G987). The products were cloned into *Bam*HI and *Eco*RI sites of pBluescript KS+, and subsequently into the insect cell expression vector pVL1393 (PharMingen), and sequenced in full. A truncated, secreted construct (amino acid residues 46–353) was prepared using primer pair HCRS124 (5'-CCCAAGGAGAAAGGGCAGCTC) and HCRS125 from a P<sub>1</sub>+ phenotype individual (165), and the sequence confirmed as described above. The products were cloned into the expression vector pAcGP67A (PharMingen). The variants of plasmids pVL- $\alpha 4$ Gal-T1-full and pAcGP67- $\alpha 4$ Gal-T1-sol were co-transfected with Baculo-Gold™ DNA (PharMingen), and virus amplified as described previously (18). Standard assays were performed in 50- $\mu$ l reaction mixtures containing 25 mM cacodylate (pH 6.5), 10 mM MnCl<sub>2</sub>, 0.25% Triton X-100, 100  $\mu$ M UDP-[<sup>14</sup>C]Gal (10,000 cpm/nmol) (Amersham Pharmacia Biotech), and the indicated concentrations of acceptor substrates (Sigma and Dextra Laboratories Ltd.) (see Table I for structures). The full coding constructs were assayed with 1% Triton X-100 homogenates of cells twice washed in phosphate-buffered saline or resuspended microsomal fractions.

**Expression of  $\alpha 4$ Gal-T1 in P<sup>k</sup>-negative Namalwa Cells**—The three full coding constructs, 67, 45, and p5, were cloned into pDR2 (CLON-TECH). Insert was excised from pBKs with *Bam*HI/*Xho*I and inserted

<sup>2</sup> R. Steffensen, J. Wiels, E. P. Bennett, and H. Clausen, unpublished observation.

P<sup>k</sup>  $\alpha$ 4Gal-T

HCRS131	
1	ATGTCCAGCCCCCGACCTCTGCTGCGGCTGCTCCGGGCGCCCCAAGGCAGCGGGTCTGCACCTGTTCATCATCGGCTTCAAGTTCACGTTTTCTCTCCATCATGATCTACTGG
1	M S K P P D L L L R L L R G A P R Q R V C T L F I I G F K F T F P V S I M I Y W
HCRS124	
121	CACGTTGTGGGAGAGCCCAAGGAGAGGGGAGCTCTATAACCTGCCAGCAGAGATCCCTGCCCCACCTTGACACCCCCACCCACCCCTCCCGGCCCCACTCCAGGCAACATCTTC
41	H V V G E P K E K G Q L Y N L P A E I P C P T L T P P T P P S H G P T P G N I F
241	TTCTGGAGACTTCAGACCGGACCAACCCCACTTCTGTTTCATGTGCTCGGTGGAGTGGCGCGCAGAACTCACCACCAATCCACGTGCTGGTCTGTGATGAAAGGGCTTCGGGTGGC
81	F L E T S D R T N P N F L P M C S V E S A A R T H P E S H V L V L M K G L P G G
361	AACGCTCTCTGCCCCGGCACCTGGGCATCTCACTTCTGAGTGTCTCCGSAATGTCCAGATGCTCCCGTGGACCTCGGGAGCTGTTCCGGGACACACCCCTGGCGACTGGTACGGC
121	N A S L P R H L G I S L L S C F P N V G Q M L P L D L R E L F R D T P L A D W Y A
481	GCCGTGACGGGCGCTGGGAGCCCTACCTGCTCCCGTGTCTCCGACGCTCCAGGATCGCACTCATGTGGAAGTTCGGCGGCATCTACCTGGACACGGACTTCATTGTTCTCAAGAAC
161	A V Q G R W E P Y L L P V L S D A S R I A L M W K F G G I Y L D T D F I V L K N
601	CTGCGGAACCTGACCAACGTGCTGGGACCCAGTCCCGCTACGTCTCAACGCGCGTCTCTGGCTTCGAGCGCGGACGAGTTCATGGCGTGTGATCGGGACTTCGTGGACAC
201	L R N L T N V L G T Q S R Y V L N G A F L A F E R R H E F M A L C M R D F V D H
721	TACAACGGCTGGATCTGGGTTCACAGGGCCCGCAGCTGCTCAGCGGGTCTTCAAGAGTGGTGTTCATCCGACGCTGGCGGAGAGCCGCGCTCGCGGGCGCTCACCCCTGCCC
280	Y N G W I W G H Q G P Q L L T R V F K K W C S I R S L A E S R A C R G V T T L P
841	CCTGAGCCTTCTACCCCATCCCTGGCAGGACTGGAAGAAGTACTTTGAGGACATCAACCCGAGGAGTCCCGCGGCTGCTCAGTGCCACCTATGCTGTCCAGCTGTGGAACAAGAAG
281	P E A F Y P I P W Q D W K Y F D I N A P E L P R L L S A T Y A V H P T V W N K K
HCRS131	
961	AGCCAGGCGACGCGTTCGAGGCCAGTCCAGGGCACTGTGGCCAGCTGCATGCCGCTACTGCCACGACGACGAGGCCATGAAATGTACTTGTGAGGGCCCCCGCAGGTACCC
321	S Q G T R F E A T S R A L L A Q L H A R Y C P T T H E A M K M Y L *
TCCCCAAGCTGCTCTGATGGGCACTGGGCGCCCTTCCCGGGAGGCAAGATTGAGGGCCCGGAGAGGGAGGCGGAGCTGCCACGGGCTTAGGACAGGCTGTTGAGGAGCTGTGGG	
AGCAGGCCAGTGGGAGGCTGTGACACCCCGAGGACAGTGTCTGTCTCGAGGAGGGCTGACACATGTGTCATAGCCAGCGGAGGGCGCTCAGTGAGTCCCGGGCTCTAGACA	
ACAGGCAGGAAGGATGAACCTCAGGGCAACCCAGGTGGTGGCAAGCCAGGAGTGTGGGACAGAGGTGCCACGAGGGCAGAGGCCGCTGTAAGGGATGGGGAAGAAGGACAAGA	
TTCCGAGAGGAGAGGAGGCTGTTGTTAGGAAGTGGCAGGGCTGGGGAGACCCAGCCCAAGGGTCCGGGGCGAGGATGCTTTGTTCTTTCTGTTTGGTTCTCTTTCGCGGG	
GGGTGGGGAGGTCACAGGGACTAGTGGGCGAGGCCAGGAAGTCCAGCTGGGGAGCCGTTTGGGGGACGCCCTTCTGCCACCCCATCTCTCTCTCCAGAGATGCCAGG	
GGGCGCTGTATGCTCTGCCCTTCCCTCAGACAGGGGCTGGGTGGGAGGCTCTTAGGCTCAGGAGAAGCATTTAAGAAACCCCAACCTTCCCGCCGATTATAACACAGGAGAA	
TAATCAATAGATAAA	

FIG. 1. Nucleotide sequence and predicted amino acid sequence of human  $\alpha$ 4Gal-T1. The amino acid sequence is shown in single-letter codes. The hydrophobic segment representing the putative transmembrane domain is underlined with a double line (Kyte and Doolittle, window of 8 (Ref. 51)). One consensus motif for N-glycosylation is indicated by asterisks. The locations of the primers used for preparation of the expression constructs are indicated by single underlining. A potential polyadenylation signal is indicated in boldface underlined type.

into the *Bam*HI/*Sal*I sites of pDR2. Transient transfection of  $5 \times 10^6$  Namalwa cells with 20  $\mu$ g of cDNA was done by double-pulse electroporation using an Easy-cell ject+ (Eurogentec). Expression of CD77/P<sup>k</sup> antigen was evaluated by fluorescence-activated cell sorting analysis on a FACSCalibur (Beckton-Dickinson) using 1A4 monoclonal antibody (19).

**Characterization of the Product Formed with  $\alpha$ 4Gal-T1**—For product characterization, 2 mg of CDH was glycosylated with a microsomal fraction of High Five cells infected with pVL- $\alpha$ 4Gal-T1-full (67) using thin layer chromatography to monitor reaction progress. The reaction products were purified on an octadecyl-silica cartridge (Bakerbond, J.T. Baker), deuterium exchanged by repeated addition of CDCl<sub>3</sub>-CD<sub>3</sub>OD 2:1, sonication, and evaporation under dry nitrogen, and then dissolved in 0.5 ml of Me<sub>2</sub>SO-*d*<sub>6</sub>, 2% D<sub>2</sub>O (20) (containing 0.03% tetramethylsilane as chemical shift reference) for NMR analysis. One-dimensional <sup>1</sup>H NMR spectra were acquired at 35 °C on a Varian Inova 600 MHz spectrometer; 1200 free induction decays were accumulated, with solvent suppression by presaturation pulse during the relaxation delay. Spectra were interpreted by comparison to those of relevant glycosphingolipid standards acquired under virtually identical conditions, as well as to previously published data for which a somewhat different temperature (65 °C) was employed (20, 21).

**Northern Analysis**—The cDNA fragment of full coding  $\alpha$ 4Gal-T1 (67) was used as probe. The probe was random primer-labeled using [ $\alpha$ <sup>32</sup>P]dCTP and a Strip-EZ DNA labeling kit (Ambion). Multiple tissue northern (MTN-H12) blot was obtained from CLONTECH. Eight human cell lines (Ramos, MutuI, BL2, Namalwa, Remb1, 8866, T51, and K562) were analyzed because P<sup>k</sup> synthase activity and antigen expression have been characterized previously (22, 23). Total cellular RNA was extracted from cell lines using the RNeasy Midi kit (Qiagen SA).

## RESULTS

**Identification and Cloning of Human P<sup>k</sup>  $\alpha$ 4Gal-T1**—Nakayama *et al.* (17) recently reported the cloning of a novel human  $\alpha$ 4GlcNAc-transferase responsible for the synthesis of the structures GlcNAc $\alpha$ 1-4Gal $\beta$ 1-4GlcNAc $\beta$ 1-R and GlcNAc $\alpha$ 1-4Gal $\beta$ 1-3GalNAc $\alpha$ 1-R. The gene was mapped to chromosome 3p14.3. Since this is the first mammalian glycosyltransferase gene available that forms an  $\alpha$ 1-4 linkage, we hypothesized that it would represent one member of a family of homologous glycosyltransferase genes. A characteristic feature of homologous glycosyltransferase genes is that different mem-

bers may encode enzymes which have different donor or acceptor sugar specificities, but the nature of the linkage formed is often retained (24). BLAST analysis of data bases using the coding region of the  $\alpha$ 4GlcNAc-transferase identified a sequenced BAC clone containing an open reading frame of 1059 bp. The coding region depicts a type II transmembrane protein of 353 amino acids with 35% overall sequence similarity to human  $\alpha$ 4GlcNAc-T (Figs. 1 and 2). The two genes show conservation of a DXD motif (25), and spacings of five cysteine residues. The predicted coding region of  $\alpha$ 4Gal-T1 has a single initiation codon in agreement with Kozak's rule (26), which precedes a sequence encoding a potential hydrophobic transmembrane segment (Fig. 1).

**Genetic Polymorphism of P<sup>k</sup>  $\alpha$ 4Gal-T1**—Sequence analysis of the  $\alpha$ 4Gal-T1 gene from six p phenotype individuals from northern Sweden revealed only one single homozygous missense mutation T548A leading to the change of residue 183 from methionine to lysine. This substitution is a few amino acid residues from the functionally important DXD motif (25). Although residue 183 is not invariant among  $\alpha$ 4Gal-T1 and the  $\alpha$ 4GlcNAc-T (M/I), the non-conservative substitution to a charged lysine residue may be expected to affect the function of the gene product. The finding that all six p individuals only revealed one missense homozygous mutation and this was not found in 12 P<sub>1</sub>+/- individuals strongly indicated that the gene identified was the P<sup>k</sup> gene. Because  $\alpha$ 4Gal-T1 was located to the same chromosomal region (22q13.2) where the P<sub>1</sub> polymorphism has been linked (22q12-ter), it was likely that it also represented the P<sub>1</sub> synthase. Analysis of the  $\alpha$ 4Gal-T1 gene in P<sub>1</sub>+ and P<sub>1</sub>- individuals, revealed two silent and one missense mutation; however, none of these showed association with the P blood group phenotype (Table II). This was confirmed by genotyping of 82 individuals, 31 P<sub>1</sub>+ and 51 P<sub>1</sub>-, where no significant correlation of the 109A and the 109G allele was observed (Table III). The PCR-based *Rca*I restriction enzyme analysis was confirmed by Southern blot analysis of P<sub>1</sub>+/- individuals (Fig. 3). The more common allele of the missense

$\alpha$ 4Gal-T1 : MSKPPD L L R L R G A P R Q R C T F I I T K E T F V S I M I Y W H V V G E P E K G Q Y N T A E I P C P T T R P T P : 69  
 AGlcNAc-T : M R R E L Q S S S - - - - - T L L V C S F L Y Q T L - - - - - S S - C F C L S F K S H Q G S A L L S : 47

$\alpha$ 4Gal-T1 : P S H P T P G N I F L E T S D T R H N F F M S V E S A A R T H P E S H L V I M K L P G G N - - A L R R H L G I L S C F : 136  
 AGlcNAc-T : H E R S - - - - - I N F L E T S E R M E P H V S C V E S A A R I Y G E N F V T F M K G L T D S T P M S S N T Y E A P F P S A I : 111

$\alpha$ 4Gal-T1 : P V I Q H L L L L R E F R D T E L A D N Y A A V Q G S W E P Y L P V L E A S F I L M N R F G G I Y D T P F L K L N L R N L T : 206  
 AGlcNAc-T : D V I F L F L D L M R R L E D T E I F S A N Q I N A S A G R N W H I S D D A S F L A I N K F G L Y N D T E V E I R I F I E E N : 150

$\alpha$ 4Gal-T1 : N V L G T Q S A T V L G A S L A F E R R E S M A L A D E V E H Y N S W I W H Q C Q L L I F F K K C C I R S L A S - - R A : 272  
 AGlcNAc-T : F L A A Q A S R Y S S G I G S L P H P F L W E C E N E V S H Y N S A I W G N G G E K K T P L R V A C K L E D F C V S D L R : 248

$\alpha$ 4Gal-T1 : R G V T T P E E A T Y P P W Q D R K K T E F D I N P E L F A L L S A T A N V V N K K S C T R F E A T S R A L A S H A : 339  
 AGlcNAc-T : L N I S F R Q Q S Y P I S Y R E R R Y F V W D T P S F N V S A P L N H H M N Q E R A V I R G E N T E V E N Y R : 314

$\alpha$ 4Gal-T1 : R Y S T H E A M K M Y L : 353  
 AGlcNAc-T : K R A R Y R D L I K P E G S V T G E L G P G N K : 341

FIG. 2. Multiple sequence analysis (ClustalW) of human  $\alpha$ 4Gal-T1 and  $\alpha$ 4GlcNAc-T. Introduced gaps are shown as dashes, and aligned identical residues are black boxed. The two amino acid substitutions (M37V and M183K) are indicated above the  $\alpha$ 4Gal-T1 sequence. Conserved cysteine residues are shown by asterisks.

TABLE II  
 Sequence polymorphisms identified in the coding region of  $\alpha$ 4Gal-T1 in  $P_1+$ ,  $P_1-$ , and  $p$  blood group individuals

Donor no.	Phenotype	nt 109 (Met <sup>37</sup> → Val)	nt 548 (Met <sup>183</sup> → Lys)	nt 903 (silent Pro <sup>585</sup> )	nt 987 (silent Thr <sup>613</sup> )
165	$P_1+$	A/G	T	G	A/G
167	$P_1+$	A/G	T	G	A/G
178	$P_1+$	A/G	T	G	A/G
183 <sup>a</sup>	$P_1+$	A	T	G/C	G
168	$P_1+$	A	T	G/C	G
173	$P_1+$	G	T	G	A
194 <sup>a</sup>	$P_1+$	G	T	G	A
332	$P_1-$	G	T	G	A
174	$P_1-$	A	T	C	G
200	$P_1-$	A	T	C	G
300 <sup>a</sup>	$P_1-$	A	T	G/C	G
321 <sup>a</sup>	$P_1-$	G	T	G	A
1	$p$	A	A	G	A/G
2	$p$	A	A	G	G
3	$p$	A	A	G	G
4	$p$	A	A	G	G
5	$p$	A	A	G	G
6	$p$	A	A	G	G

<sup>a</sup> Indicates that the sequence obtained by direct sequencing of PCR products were confirmed on cloned products.

TABLE III  
 Correlation of the missense polymorphism with  $P_1+/-$  blood group phenotype

Genotyping was performed by *RcaI* restriction analysis of PCR products.

Phenotype	Genotype (nt 109)	Cases	Allele frequencies	
			109A	109G
$P_1+$	AA	11	0.63	0.37
	AG	17		
	GG	3		
$P_1-$	AA	32	0.79	0.21
	AG	17		
	GG	2		

mutation at A109G encodes a methionine at residue 37 in the C-terminal part of the putative hydrophobic signal sequence (Figs. 1 and 2). The conservative substitution of residue 37 to valine is not predicted to change the catalytic activity or affect retention in the Golgi.

**$\alpha$ 4Gal-T1 Encodes Exclusive  $P^k$   $\alpha$ 4Gal-T Activity**—Expression of full coding constructs of  $\alpha$ 4Gal-T1<sup>37M</sup> and  $\alpha$ 4Gal-T1<sup>37V</sup> in insect cells resulted in marked increase in galactosyltransferase activity with CDH, compared with uninfected cells or cells infected with a control construct (Fig. 4). In contrast, no activity was found with the  $\alpha$ 4Gal-T1<sup>183K</sup> gene from  $p$  individuals. Importantly, neither  $\alpha$ 4Gal-T1<sup>37M</sup> or  $\alpha$ 4Gal-T1<sup>37V</sup> constructs conferred  $\alpha$ 4Gal-T activity with the neolacto-series

(paragloboside) glycolipid acceptor for  $P_1$  synthase activity (Fig. 4). The assay conditions for measuring  $P^k$  and  $P_1$  synthase activity was the same except substitution of the acceptor substrate, and these conditions were previously used to demonstrate both activities in kidney extracts from  $P_1+$  and  $P_1-$  individuals (11). The soluble, secreted construct encoding residues 47–353 did not result in active  $\alpha$ 4Gal-T activity (data not shown). Attempts to obtain complete conversion of CDH to CTH were unsuccessful, but a one-dimensional <sup>1</sup>H NMR spectrum of the purified reaction mixture (data not shown) clearly exhibited H-1 resonances diagnostic for CTH at levels approximately 30% of those of the CDH acceptor substrate. Thus, in addition to major resonances at 4.205 ppm (<sup>3</sup> $J_{1,2}$  = 7.2 Hz) and 4.165 ppm (<sup>3</sup> $J_{1,2}$  = 7.9 Hz), corresponding to H-1 of Gal $\beta$ 4 and Glc $\beta$ 1 of CDH, minor resonances were observed at 4.794 ppm (<sup>3</sup> $J_{1,2}$  = 3.7 Hz) and 4.258 ppm (<sup>3</sup> $J_{1,2}$  = 6.9 Hz), corresponding to H-1 of Gal $\alpha$ 4 and Gal $\beta$ 4 of CTH (the chemical shift of Glc $\beta$ 1 H-1 is not affected by the addition of the terminal Gal $\alpha$ 4 residue). The chemical shift and <sup>3</sup> $J_{1,2}$  coupling of the downfield H-1 resonance are particularly characteristic for Gal $\alpha$ 4 of CTH and other globo-series glycosphingolipids (20, 21). Analysis with a number of saccharide acceptors including lactose, lactosamine, and benzyl  $\beta$ -lactoside revealed no significant activity over background values (data not shown).

**Expression Pattern of  $\alpha$ 4Gal-T1**—Northern analysis with mRNA from 12 human organs revealed a ubiquitous expression pattern with high expression in kidney and heart and low

FIG. 3. *RcaI* genotyping of position A109G by Southern analysis. DNA from five phenotyped donors was digested with restriction enzymes as indicated, and the blot probed with the full coding  $\alpha$ 4Gal-T1 (67) construct. The *RcaI* digestion confirmed the PCR-based genotyping presented in Table II. The *EcoRI* polymorphism found in individuals 165 and 183 is outside the coding region of  $\alpha$ 4Gal-T1 and is unrelated to the  $P_1$  phenotype.

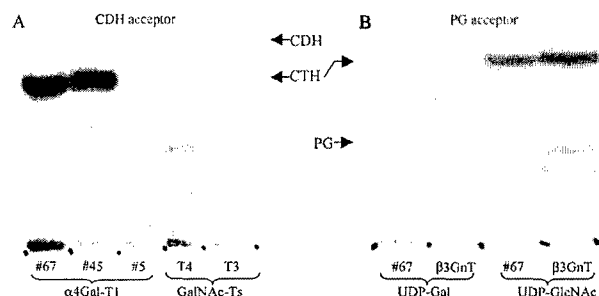
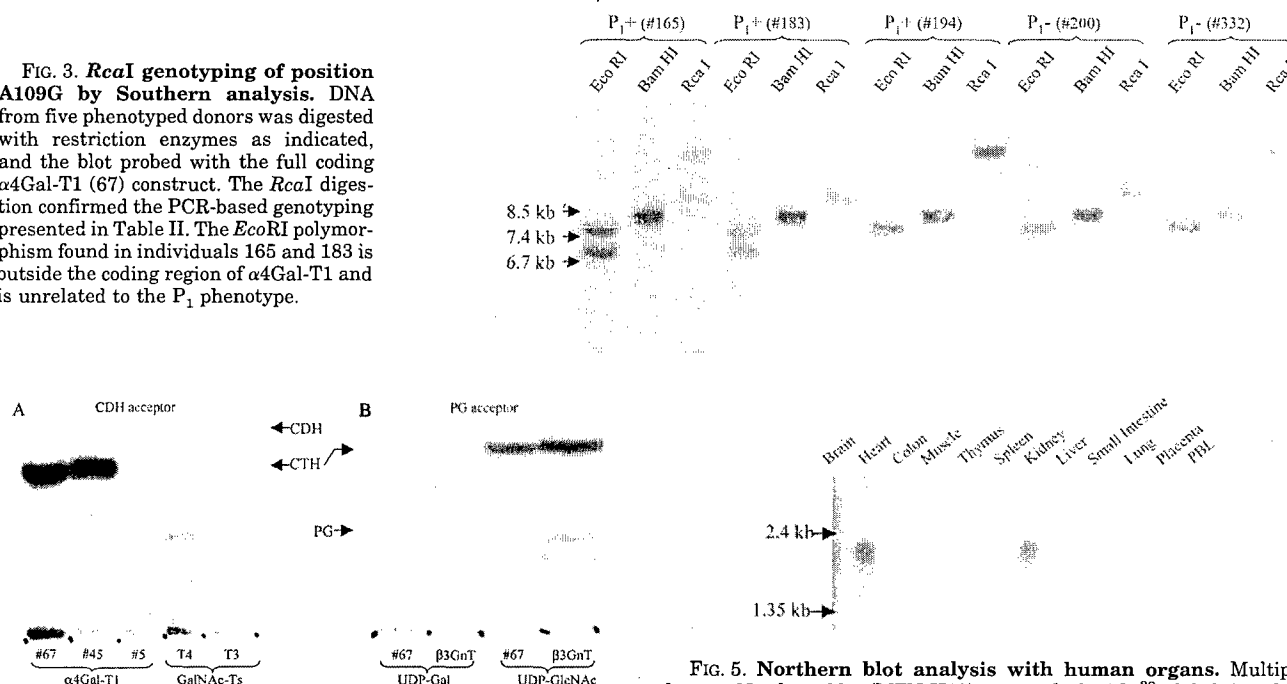


FIG. 4. Expression of full coding  $\alpha$ 4Gal-T1 variants in High Five cells. Assays were performed with microsomal fractions, and controls included constructs encoding polypeptide GalNAc-T3 and -T4 (52), as well as a  $\beta$ 3GlcNAc-T (24). Autoradiography of high performance thin layer chromatography of reaction products (4 h) purified by SepPack C-18 columns. Panel A,  $p^k$  assay using 25  $\mu$ g of CDH as substrate. Plate was run in chloroform-methanol-water (60/35/8, v/v/v). Constructs from the two different alleles identified from  $P_1$ +/− individuals (45 and 67) resulted in  $\alpha$ 4Gal-T activity toward CDH, while the construct derived from p (5) showed no activity above background found with control constructs. Panel B,  $P_1$  assay using 20  $\mu$ g of paragloboside (PG) as substrate. Plate was run in chloroform-methanol-water (60/40/10, v/v/v). No specific product was formed with UDP-Gal donor substrate, whereas the  $\beta$ 3GlcNAc-T transferred GlcNAc into paragloboside with UDP-GlcNAc. Considerable GlcNAc-T activity was observed in both 67 and  $\beta$ 3GnT microsomal fractions, yielding a GlcNAc-CTH-related product.

expression in other organs (Fig. 5). Kidney primarily synthesizes globo-series glycosphingolipids (27). Analysis of eight human cell lines revealed an expression pattern correlating with  $\alpha$ 4Gal-T1 activity and cell surface expression of  $P^k$  antigen (Fig. 6) (22, 23). Ramos cells have the highest antigen expression and  $\alpha$ 4Gal-T activity, and strong expression of  $\alpha$ 4Gal-T1. In contrast, Namalwa cells, which do not produce  $P^k$  antigens and have no measurable  $\alpha$ 4Gal-T activity, showed no expression of  $\alpha$ 4Gal-T1. However, transient transfection of Namalwa cells with the full coding constructs of  $\alpha$ 4Gal-T1 (67 and 45) resulted in  $P^k$ /CD77 expression as revealed by fluorescence-activated cell sorting analysis (Fig. 7).

#### DISCUSSION

The  $\alpha$ 4Gal-T1 gene characterized in this report provides a molecular genetic basis for the rare p histo-blood group phenotype found in Västerbotten County, in the northern part of Sweden (28). A single inactivating homozygous missense mutation in the catalytic domain of the enzyme was found in all six p phenotype individuals studied. We have previously characterized erythrocyte  $PP^k$  antigen expression and  $\alpha$ 4Gal-T activity in EBV-transformed cells from two of these individuals (15), and found a complete deficiency of  $P^k$  antigen and  $\alpha$ 4Gal-T activity. Iizuka *et al.* (29), reporting essentially the same experiment, suggested that a catalytically active  $P^k$  transferase was indeed expressed in p individuals, as evidenced by  $P^k$  synthase activity in EBV-transformed cells; however, in ac-

FIG. 5. Northern blot analysis with human organs. Multiple human Northern blot (MTN-H12) was probed with  $^{32}$ P-labeled  $\alpha$ 4Gal-T1 probe. kb, kilobases.

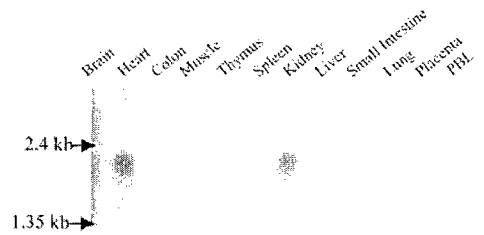
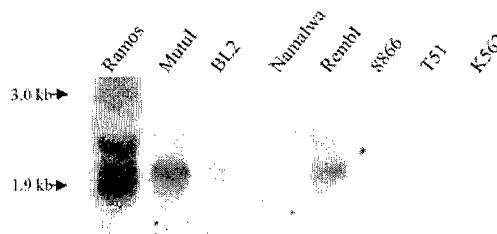


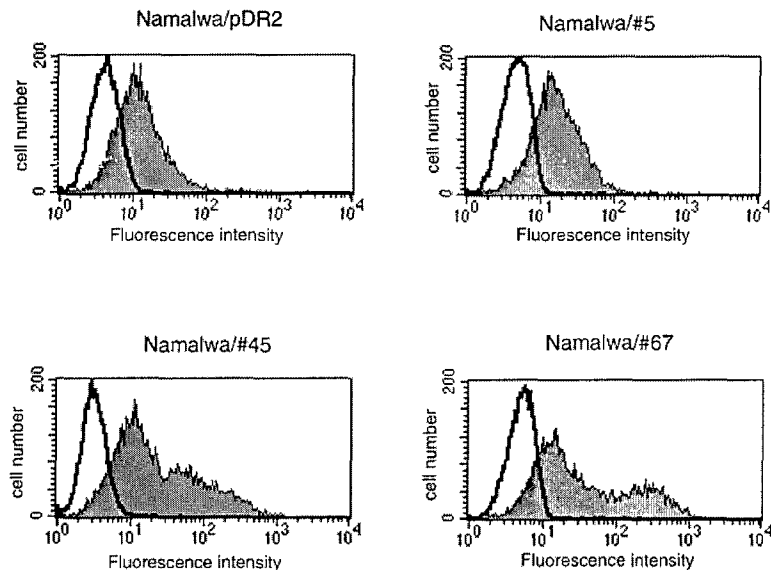
FIG. 6. Northern blot analysis with eight human B cell lines. Transcript sizes are approximately 2 and 3 kilobases (kb).



cordance with the proposed p phenotype of the individual studied, the transformed cells did not express  $P^k$  antigen. This led Iizuka *et al.* (29) to suggest that p phenotype individuals carry a functionally active  $P^k$   $\alpha$ 4Gal-T gene, and that the p phenotype was a result of an yet unknown epigenetic mechanism. The data presented here are not in agreement with this, and support a simple allelic model with an active  $P^k$  and an inactive p allele. It is, however, possible that the p phenotype in different populations has a different molecular genetic basis. The molecular genetics of all other characterized histo-blood group systems defined by carbohydrate antigens, *i.e.* ABO (30), Hh (31), Sese (32), and Lewis (33, 34), have been shown to adhere to a model with simple inactivating mutations of glycosyltransferase genes.

The presented data, however, do not explain the molecular genetic basis of the  $P_1$  blood group polymorphism. Although, the  $P_1$  polymorphism is linked to the same chromosomal localization as  $\alpha$ 4Gal-T1, we found no genetic polymorphisms in the  $\alpha$ 4Gal-T1 gene associated with the  $P_1$ +/− phenotypes, and recombinant  $\alpha$ 4Gal-T1 variants did not express  $P_1$  synthase activity *in vitro* (Tables II and III, Fig. 4). Searching the available chromosome 22 sequence did not reveal additional homologous genes. Therefore, the following possibilities exist: (i)  $\alpha$ 4Gal-T1 can be activated by another non-homologous polymorphic gene or gene product and function as a  $P_1$  synthase; (ii) a second polymorphic  $\alpha$ 4Gal-T gene, which is non-homologous to  $\alpha$ 4Gal-T1, exists; or (iii) an alternatively spliced version of  $\alpha$ 4Gal-T1 encodes a form capable of functioning as a  $P_1$  syn-

FIG. 7. Cell surface expression of  $P^k$ /CD77 antigen in Namalwa cells after transient transfection of  $\alpha 4$ Gal-T1. Constructs p5, 45, and 67 as well as empty pDR2 vector were electroporated in Namalwa cells, and expression of  $P^k$ /CD77 antigen was tested after 48 h. Cells were labeled with 1A4 monoclonal antibody and goat anti-mouse-fluorescein isothiocyanate (gray histograms) or with goat anti-mouse-fluorescein isothiocyanate alone (empty histograms) and analyzed with a FACSCalibur flow cytometer. Strong labeling with 1A4 was found only with constructs 45 and 67.



thase. The first possibility has a precedent in two members of the  $\beta 4$ Gal-T gene family,  $\beta 4$ Gal-T1 and -T2, both of which are modulated by  $\alpha$ -lactalbumin to change their function from *N*-acetylglucosaminyl synthases to lactose synthases (35–37). Binding of  $\alpha$ -lactalbumin to these galactosyltransferases changes the acceptor substrate specificity from GlcNAc to Glc, but also to some degree affects the donor substrate specificity to include UDP-GalNAc (38). The induction of  $\beta 4$ Gal-T1 by  $\alpha$ -lactalbumin to enable it to function as a lactose synthase is combined with a complex regulatory mechanism by which the  $\beta 4$ Gal-T1 synthase is 100-fold up-regulated in mammary glands (39). As lactose is the major nutrient in milk, this complex model for its synthesis appears to be in accordance with the biological function. The  $P_1$  antigen has only been detected as a minor glycosphingolipid component, and no biological function for this polymorphic antigen has been identified. At present, therefore, it may seem less likely that a unique modulator of the  $\alpha 4$ Gal-T1 gene has evolved. The second possibility of the existence of another polymorphic non-homologous  $\alpha 4$ Gal-T gene located in the same chromosomal region implies that the encoded  $\alpha 4$ Gal-T functions as both  $P^k$  and  $P_1$  synthases. This is based on the findings that  $p$  individuals do not produce  $P_1$  antigens, and it is supported by the finding that erythrocytes of  $P_1$  individuals contain relative less LacCer and more Gb<sub>3</sub> than  $P_2$  individuals (40). Generally, glycosyltransferases with similar functions are encoded by homologous glycosyltransferase gene families (24); however, recently two non-homologous  $\beta 3$ GlcNAc-transferases both functioning as poly-*N*-acetylglucosaminyl synthases have been identified (41, 42). The third possibility is unlikely, as the coding region of  $\alpha 4$ Gal-T1 was found in a single exon. However, there are precedents for multiple protein forms originating from such genes by differential usage of splice donor sites of introns placed in 3'-UTR (43). Although the Northern analysis with mRNA from human organs only revealed one transcript of 2.3 kilobases (Fig. 5), the Northern analysis with total RNA from human cell lines may suggest the presence of additional larger transcripts (Fig. 6). The nature of these transcripts required further analysis to explore the possibility that they may encode a novel form of the  $\alpha 4$ Gal-T1 protein with  $P_1$  synthase activity. A possible mechanism for the  $P_1$  blood group polymorphism would include sequence polymorphisms in the 3'-UTR directing different donor splice sites in the 3' coding region of the characterized  $\alpha 4$ Gal-T1 coding sequence. It is important to note, however, that a

total of three polymorphisms were identified in  $P_1$ +/- individuals (Table II), and none of these correlated with the phenotype. This finding argues against the existence of another polymorphism in this gene (3'-UTR) responsible for the  $P_1$ +/- polymorphism, because it would be expected that at least some of these would have arisen after the  $P_1$ +/- polymorphism and hence co-segregate with this as found e.g. for the polymorphisms in the ABO gene locus (30).

$\alpha 4$ Gal-T1 is homologous to an  $\alpha 4$ GlcNAc-T located at 3p14.3 (17). The  $\alpha 4$ GlcNAc-T forms the linkage GlcNAc $\alpha$ 1-4Gal $\beta$ 1-3/4R, where R can be GalNAc, GlcNAc, or less effectively, glucose. Preference for mucin oligosaccharides of the core 2 structure was found, and the gene was shown to control expression of Con-A-binding class-III mucins in stomach and pancreas. Genetic polymorphisms in expression of the  $\alpha 4$ GlcNAc structures have not been reported. The sequence similarity with  $\alpha 4$ Gal-T1 (35% overall amino acid sequence similarity) is similar to that found among other homologous glycosyltransferases with similar functions, and the characteristic feature of conserved spacings of cysteine residues (five cysteine residues align, Fig. 2) is also found. Both enzymes transfer to galactose, but, whereas the acceptor disaccharide specificity of the  $\alpha 4$ GlcNAc-T appears to be broad,  $\alpha 4$ Gal-T1 is apparently highly specific for the glycolipid, lactosylceramide. Lopez *et al.* (44) recently characterized an  $\alpha 4$ Gal-T activity in insect cells and found it had preferred acceptor substrate specificity for Gal $\beta$ 1-3GalNAc $\alpha$ 1-R rather than lacto-series structures. Thus, the acceptor substrate specificity is similar to that of the  $\alpha 4$ GlcNAc-T and different from  $\alpha 4$ Gal-T1.

The *N. gonorrhoeae* *lgtC*  $\alpha 4$ Gal-T (12) exhibits no significant sequence similarity to the  $\alpha 4$ Gal-T1 reported here. However, two human genes homologous to *lgtC* were identified with significant sequence similarities and conserved DXD motif (data not shown). We have been unable to demonstrate glycosyltransferase activity with these two human genes using full coding and secreted recombinant constructs expressed in insect cells. Nevertheless, they may represent glycosyltransferase genes and encode  $\alpha 4$ Gal-Ts, but they are unlikely to be involved in the  $P_1$  polymorphism as they are not located on chromosome 22. One of the genes is located at 8q24 distal to the *c-myc* oncogene, and hence part of region of chromosome 8, which is translocated in Burkitt's lymphoma. Since Burkitt's lymphoma is associated with high level expression of  $P^k$  (45), this represented a candidate for the  $P^k$  gene. However, North-

ern analysis of various B cell lines (Fig. 6) shows that  $\alpha$ 4Gal-T1 expression clearly correlates with P<sup>k</sup> synthase activity and antigen expression in Burkitt's lymphoma cell lines (22, 23).

The P blood group system is implicated in important biological phenomena. Blood group p individuals have strong anti-P<sub>1</sub>PP<sup>k</sup> IgG antibodies and these are implicated in high incidence of spontaneous abortions (46). The globo-series glycolipid antigens constitute major receptors for microbial pathogens with the Gal $\alpha$ 1-4Gal linkage being an essential part of the receptor site (for a review, see Ref. 47). The P<sup>k</sup> glycolipid is the CD77 antigen, a B cell differentiation antigen, which is able to transduce a signal leading to apoptosis of the cells (48). Furthermore, the association of this glycolipid with the type I interferon receptor or with the human immunodeficiency virus type 1 co-receptor, CXCR4, seems to be crucial for the functions of these receptors (49, 50). Cloning of the P<sup>k</sup> synthase is an important step toward understanding the biological roles of the globo-series class of glycolipids, and a first step in elucidating the molecular genetics of the P blood group system.

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21 Feb 03

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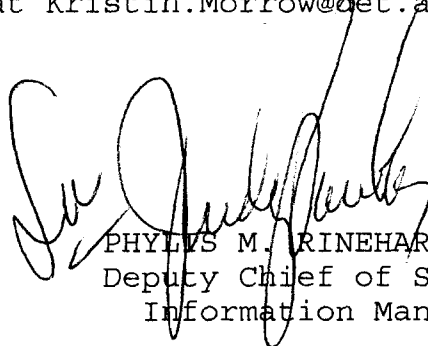
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